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(54) Title: NOVEL ANTIBODY-CYTOKINE FUSION PROTEIN, AND METHODS OF MAKING AND USING THE SAME

(57) Abstract

By fusing a cytokine (e.g., IL-2) to an antibody (e.g. IgG3), a molecule has been created with the functional characteristics of both proteins. The pharmacokinetic properties of such a fusion protein may be greatly improved over those of cytokine alone (e.g., IL-2) and previously described antibody-IL-2 fusions. The molecule is intact and recoverable from the blood of mice hours after intraperitoneal injection. The present fusion protein also reaches distant organs throughout the animal. The 7-hour half-life *in vivo* of an exemplary IL2-IgG3 molecule is much longer than that of IL-2 and may make it more useful than IL-2 for multiple *in vivo* applications. Other IL-2 fusion proteins used as vaccines have been shown to elicit an increased immune response against the fused protein and have been studied for both prevention and treatment against tumors and viruses expressing those antigens. The exemplary IgG3-IL2 fusion protein binds a hapten that can be conjugated to most antigens of interest. Antigens can therefore be linked to bioactive IL-2 without the complexities and uncertainties of making IL-2 fusions with each antigen individually. This approach has been tested using BSA as a model antigen. The antibody response to IgG3-IL2-bound BSA is increased over that of BSA or IgG3-bound BSA. This system should be useful in potentiating the immune response to antigen and in screening antigens for use in vaccines.

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TITLE OF THE INVENTION

NOVEL ANTIBODY-CYTOKINE FUSION PROTEIN, AND METHODS OF MAKING AND USING THE SAME

The present invention was developed at least in part by grants CA-16858 and AI-29470 (from the National Institutes of Health) and DHHS PHS National Institutional Research Service Award T 32 CA09056. Thus, the U.S. government may have certain rights with regard to the present invention.

BACKGROUND OF THE INVENTION

Field of the Invention:

The present invention concerns an anti-hapten/IL-2 fusion protein and methods of producing and using the same, and more particularly to a method for targeting either (a) the antibody or hapten thereof (i.e., antigenic binding partner) to a cell bearing a cytokine receptor on its surface (e.g., T cells) or (b) the cytokine to a cell bearing the hapten of the antibody on its surface.

Discussion of the Background:

The therapeutic value of Interleukin 2 (IL-2) is limited by its short half life and systemic toxicity. One approach to overcoming these problems is to fuse this protein to an antibody, a protein with a long half life and the ability to target a unique antigen within the body.

Interleukin-2 (IL-2) is an important cytokine involved in the generation of an effective cell-mediated immune response. The activities of IL-2 include stimulation of T cells to proliferate and become cytotoxic.[1,2,3] IL-2 also stimulates cytotoxicity in natural killers (NK), macrophages and a variety of other cell types, giving rise to LAK cells.[4] These promising in vitro activities have lead to the in vivo use of IL-2 to treat cancer and Acquired Immune Deficiency Syndrome (AIDS) patients.

Two major problems limit the therapeutic value of IL-2. It is toxic when administered systematically at the appropriate dose.[5,6,7] In addition, and the short in vivo half-life of IL-2 requires multiple daily injections or continuous infusion to maintain effective doses.[8] The toxicity of systemic IL-2 administration is due to increased vascular permeability throughout the patient, leading to vascular leak syndrome. Significant improvements in the therapeutic usefulness of IL-2 might be achieved by extending its in vivo half-life to allow constant, subtoxic levels to be administered or by specifically directing it to its intended site of action, avoiding unnecessarily high systemic levels.

Monoclonal Antibodies (MAbs) have a combination of traits that have made them valuable therapeutic tools for targeting. The exquisite specificity and long half-life of MAbs enable significant amounts of an injected dose to localize to the antigen site. Radionuclides conjugated to MAbs have proven useful as diagnostic agents[9,10] and toxins conjugated to MAbs have shown potential as therapeutics.[11]

Previous studies of a fusion protein combining IgG1 and IL-2 showed that it bound antigen, stimulated proliferation and cytotoxicity, and mediated ADCC in vitro.[12,13] Experiments using severe combined immunodeficiency mice reconstituted with human LAK cells demonstrated that this IgG1-IL2 fusion protein suppressed dissemination and growth of metastases of a human neuroblastoma.[13]

Previously reported fusions differ in their specific activity. In one case, an IgG1-IL2 fusion protein was reported to be similar to IL-2 in stimulating CTLL-2 proliferation.[12] In contrast, another IgG1-IL2 fusion was reported to be 30- to 100-fold less active than IL-2.[14]

Although antibodies with greater specificity and affinity for tumor-associated antigens provide increasingly useful reagents in the diagnosis and therapy of human cancer, their ability to kill tumor cells is suboptimal. Conjugation with

toxin or radionuclide increases the ability of antibodies to kill tumors but the inaccessibility of many tumor cells within a dense tumor mass and the loss of antigen expression by rapidly mutating tumor cells make it difficult for these therapeutics alone to kill every tumor cell and effect a cure.

An alternative strategy is to use anti-tumor antibodies to activate an immune response against the tumor. An ongoing immune response should be able to access the entire tumor and should be directed against many different tumor associated antigens so that antigen modulation should not render tumor cells resistant. Indeed, the lysis of tumor cells by antibodies has been shown in some cases to activate the patient's immune system to attack and kill the remaining tumor cells.[15]

IL-2 is a potent stimulator of the immune system. Simultaneous treatment with Ab and IL-2 results in improved therapeutic outcome compared to either treatment alone, supporting the view that an increased immune response can improve upon the efficacy of antibody therapy.[16] However, the use of IL-2 as an immune stimulant is limited by its short half-life *in vivo* and its systemic toxicity.[6,17]

One approach to improving the usefulness of IL-2 is to target it to the site of the tumor, thereby increasing its effective local concentration without causing systemic toxicity. Transfection and expression of the IL-2 gene in tumor cells has been one means of achieving higher concentrations of IL-2 in the neighborhood of the tumor and has been shown to generate an anti-tumor immune response *in vivo*.[18]

Antibody-IL-2 fusion proteins have been constructed with the expectation that the antibody binding specificity may target IL-2 to the site of the tumor, reducing systemic toxicity while improving the local immune response. These fusion proteins have been shown to bind antigen[12,14] and retain antibody effector functions including the ability to stimulate

antibody dependent cell-mediated cytotoxicity and bind to Fcγ RIII.[16] The IL-2 moiety retains the ability to stimulate the proliferation of an IL-2 dependent cell line with differences in specific activity that may relate to the location of the fusion.[14]

In vitro, Ab-IL-2 fusion proteins were shown to stimulate the increased cytotoxicity of a tumor specific T cell line.[12,14] In severe combined immune deficient (scid) mice reconstituted with human LAK cells, an Ab-IL-2 fusion protein prevented tumor metastases from growing in the lungs following intravenous injection of tumor cells.[13] More recent experiments in immune competent mice with syngeneic tumors show that intraperitoneal treatment with Ab-IL-2 cured some mice and improved the overall survival rate over that of Ab treated, IL-2 treated or Ab and IL-2 treated mice (Harvill et al., unpublished results). The success of these proteins in initial animal experiments when combined with the results described herein for the present invention suggests that they may be useful as cancer therapeutics.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide a novel antibody-cytokine fusion protein which retains at least some of the half-life characteristics of the antibody (e.g., which lengthens the effective therapeutic duration of the cytokine) and at least some of the biological properties and/or activities of the cytokine.

A further object of the present invention is to provide a novel method of producing the present antibody-cytokine fusion protein.

A further object of the present invention is to provide a novel method for delivering a cytokine or moiety or fragment exhibiting at least some of the biological activity of the cytokine to a cell bearing an antigen on its surface which

binds (preferably specifically) to the antibody moiety of the present fusion protein.

A further object of the present invention is to deliver an antigen which binds specifically to the antibody moiety of the present fusion protein to a cell having a cytokine receptor on its surface (e.g., B cells, T cells, etc.).

A further object of the present invention is to provide a novel method of stimulating an immune response to an antigen, in which the antigen is dansylated, the antibody moiety is an anti-dansyl monoclonal antibody and the dansylated antigen and anti-dansyl monoclonal antibody-IL2 fusion protein are administered to an animal capable of producing an immune response (e.g., a bird such as a chicken, duck, goose or ostrich or a mammal such as a rabbit, mouse, rat, goat, horse or primate) in a dosage effective for increasing the immune response of the animal to the antigen.

A further object of the present invention is to provide a novel method for increasing the immune response of a patient having a suppressed immune system (e.g., patients being administered immunosuppressants such as those having recently undergone tissue or organ transplants, patients suffering from acquired immune deficiency syndrome, etc.) using the present method, but in which the antigen is the dansylated B cell or T cell which is uninfected by a retrovirus (e.g., human immunodeficiency virus, Epstein-Bar virus, etc.).

A further object of the present invention is to provide a novel composition for use in the present methods of stimulating an immune response to an antigen or increasing the immune response of a patient having a suppressed immune system, containing a dansylated antigen and an anti-dansyl monoclonal antibody-IL2 fusion protein (which may further contain a physiologically acceptable carrier, such as physiological saline).

These and other objects of the present invention, which will be readily understood in the context of the following

detailed description of the preferred embodiments, have been provided by a novel antibody-cytokine fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody (preferably a monoclonal antibody, more preferably an anti-dansyl monoclonal antibody), L is a covalent bond or linking group (preferably of from 1 to 10 naturally-occurring amino acids, more preferably of from 1 to 5 naturally-occurring amino acids, and most preferably a cysteine residue), and Ck is a cytokine, lymphokine (preferably selected from the group consisting of an interleukin, macrophage arming factor, lymphocyte inhibition factor, monocyte chemotactic and activating factor and granulocyte-macrophage colony stimulating factor) or biologically active fragment thereof (e.g., a fragment retaining at least 10% of the biological activity of Ck as assayed by an art-recognized assay);

and methods of making and using the same as described hereunder.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B show the exemplary IgG3-IL2 assembled and secreted as an H_2L_2 heterotetramer in TAAG cells incubated with [^{35}S]methionine as the sole source of methionine in the presence (lane 2) or absence (lane 3) of tunicamycin, an inhibitor of *N*-linked glycosylation; samples were precipitated from the cell culture supernatant with dansyl-BSA-Sepharose and analyzed by SDS-PAGE, unreduced on a 5% phosphate gel (Fig. 1A) and reduced on a 12.5% Tris-glycine gel (Fig. 1B);

Fig. 2 is a graph depicting FcγRI binding by IgG3 and IgG3-IL2, in which U937 cells stimulated with gamma Interferon were incubated with ^{125}I-labeled IgG3 and varying concentrations of unlabeled IgG3 or IgG3-IL2 as competitor;

following 3 h incubation at 13°C, receptor bound radioactivity was quantitated, and values are expressed as percentage of maximum inhibition obtained with a 200-fold excess IgG3;

Fig. 3 is a graph showing complement mediated hemolysis by IgG3 and IgG3-IL2, in which IgG3-IL2 and IgG3 were quantitated and assayed for their ability to effect complement mediated hemolysis, and results are expressed as the percentage of the total counts available;

Fig. 4 is a graph showing the stimulation of CTLL-2 cell proliferation by hrIL-2 and IgG3-IL2, in which the abilities of hrIL-2 and two separate preparations of IgG3-IL2 to stimulate the proliferation of the IL-2 dependent cell line CTLL-2 are compared; cell proliferation was measured by incorporation of [^{3}H]thymidine into newly synthesized DNA, the molar concentrations of IgG3-IL2 were based on the protein concentration and the apparent molecular weight of 200 kDa, and the concentration of hrIL-2 was given by the supplier;

Fig. 5 is a bar graph showing the generation of LAK activity by various concentrations of hrIL-2 or IgG3-IL2, in which PBL cultured for 3 days with hrIL-2 or IgG3-IL2 at the indicated doses were tested for their cytotoxicity against ^{51}Cr-loaded Raji target cells; results are expressed as the additional percentage of total ^{51}Cr specifically released by LAK cells compared to that of unstimulated PBL (average of three samples with the indicated standard deviation);

Figs. 6A, 6B and 6C are graphs showing IL-2R binding of hrIL-2 and IgG3-IL2, in which YT-1 cells were incubated at 4°C for 3 h with ^{125}I-labeled IL-2 and varying concentrations of IgG3-IL2 as unlabeled competitor to assess binding to the IL-2R; untreated cells were used to evaluate binding to the intermediate affinity IL-2R$\beta\gamma$ (Fig. 6A), and YT-1 cells pretreated with forskolin (YT-1') were used to assess binding to the high affinity IL-2R$\alpha\beta\gamma$ (Fig. 6B), and YT-1' cells were incubated with ^{125}I-labeled IgG3-IL2 and varying concentrations of IgG3-IL2 or hrIL-2 as unlabeled competitor (Fig. 6C); the

percent inhibition of binding is plotted against the concentration of the unlabeled competitor and the amount required for 50% inhibition of binding is indicated;

Figure 7 shows the half-life of ^{125}I-labeled IgG3 and ^{125}I-labeled IgG3-IL2 injected intraperitoneally into BALB/c mice, in which iodinated proteins were injected and the residual radioactivity determined over time using a whole body gamma counter (data are expressed as the percent of the initial injected radioactivity present at each time point);

Figure 8 shows the tissue distribution of ^{125}I-labeled IgG3 and ^{125}I-labeled IgG3-IL2 injected intraperitoneally into BALB/c mice, in which mice were sacrificed 4 hours after injection and the radioactivity present in selected organs determined (values are expressed as the radiolocalization index, defined as [(cpm/gram of tissue)/(cpm/gram of the entire animal)] x 100);

Figures 9(A) and 9(B) show the recovery of intraperitoneally injected ^{125}I-labeled proteins from the blood of mice, in which ^{125}I-labeled proteins were recovered from the serum of mice by precipitation with DNS-BSA-Sepharose 4 hours after i.p. injection, and recovered proteins, along with molecular weight markers, were analyzed by SDS-PAGE under non-reducing (Fig. 9(A)) and reducing (Fig. 9(B)) conditions (lane 1: IgG3-IL2 before injection; lane 2: IgG3 before injection; lane 3: rainbow molecular weight markers (Amersham, Arlington Heights, IL); lane 4: IgG3-IL2 recovered; lane 5: IgG3 recovered); and

Figure 10 shows the IgG3-IL2 stimulated antibody response to DNS-BSA-Sepharose, in which five mice per group were injected with DNS-BSA-Sepharose alone (open triangles), DNS-BSA-Sepharose bound by antidansyl IgG3 (open squares) or DNS-BSA-Sepharose bound by antidansyl IgG3-IL2 (open circles); identical booster injections were delivered on day 70 (indicated by the arrow), serum was collected at the intervals

shown, pooled within each group and analyzed by ELISA on DNS-BSA coated plates with isotype-specific secondary antibodies.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention concerns an anti-hapten/IL-2 fusion protein. In a further embodiment, the hapten is dansyl, the isotype of the antibody is human IgG3, and the IL-2 is human. However, virtually any hapten specificity can be used and the isotype and source of the antibody and the specific IL-2 can be changed without adversely impacting the general usefulness of this invention.

In addition to its anti-tumor activities, IL-2 has been used in vaccines to increase the immune response to attached antigens.[19,20,21,22,23] Although the mechanism is not well understood, IL-2 may target the molecule to particular cells for more effective antigen presentation and/or may deliver a stimulatory signal that directly or indirectly enhances the immune response to the attached antigen. Physically linking the antigen and IL-2 has been shown to be critical to maximizing the immune response to the antigen.[19]

The present fusion protein (e.g., IgG3-IL2) may be capable of binding dansyl, a hapten that can be easily linked to primary amine groups. The present fusion protein can therefore be used to potentiate the immune response to any substance that can be conjugated to dansyl. Therefore, the present fusion protein should allow a panel of antigens to be rapidly tested in order to both (a) determine the mechanism by which a cytokine (e.g., IL-2) affects the immune response, and (b) identify the protein(s) or peptide(s) that will be most useful in a therapeutic application.

Abbreviations used in this application include: IL-2, interleukin 2; IL-2R, interleukin 2 receptor; NK, natural killer; LAK, lymphokine activated killer; MAb, monoclonal antibody; Ig, immunoglobulin; C', complement; DNS, N,N dimethyl-1-aminonaphthalene-5-sulfonyl chloride; BSA, bovine

serum albumin; SRBC, sheep red blood cell; IMDM, Iscove's modified Dulbecco's medium; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CM, complete media; V, immunoglobulin variable region; C, immunoglobulin constant region; H, immunoglobulin heavy chain; L, immunoglobulin light chain; PBS, phosphate buffered saline; ELISA, enzyme linked immunosorbent assay; FcγR, Fc gamma receptor; ADCC, antibody dependent cell-mediated cytotoxicity; scid, severe combined immune deficient.

Within the fusion protein, the antibody combining site retains its ability to bind antigen, and the IL-2 retains its ability to activate the cellular immune system.

To examine the biochemical properties of an antibody-cytokine molecule, a fusion protein was constructed linking the N-terminus of human IL-2 to the C-terminus of IgG3. A similar fusion between IgG1 and IL-2 has been shown to bind antigen, generate antibody-dependent cellular cytotoxicity (ADCC) and stimulate T cell proliferation and cytotoxicity. The exemplary fusion protein of the present invention, termed IgG3-IL2, is appropriately *N*-glycosylated within the IgG3 C_H2 domain, binds the human high affinity Fc receptor (FcγRI) with an affinity slightly lower than that of IgG3, and is able to activate complement via the classical pathway to lyse antigen coated sheep red blood cells (SRBC).

The extremely short half-life of IL-2 *in vivo* poses a major obstacle to its effective use as a therapeutic.[17] The need for stable blood levels has led to difficult regimens of continuous infusion or multiple daily injections of IL-2 and has prompted efforts to extend its half-life *in vivo* by conjugating it to PEG.[24] The short half-life of IL-2 is attributed in part to its small size (15 kDa), which is below the molecular weight cut off of the kidney and is presumed to cause it to be rapidly eliminated from the blood.[8]

Fusing IL-2 to an antibody may increase its *in vivo* half-life both by increasing its size and by incorporating the

half-life characteristics of the antibody. Surprisingly, a previous fusion of IL-2 to IgG1 resulted in a molecule with a half-life of only 0.3 hours, leading to the conclusion that continuous infusion would be necessary in therapeutic applications.[25] Results with the present fusion protein (e.g., IgG3-IL2) demonstrate that this molecule has a half-life of at least 1 hour, preferably at least 2 hours, more preferably at least 4 hours and most preferably at least 7 hours, greatly extended compared to that of IL-2 or prior IgG1-IL2 fusion proteins.

For example, recovery of intact IgG3-IL2 exemplified hereunder from animals 4 hours after intraperitoneal injection showed the majority of radiation in the blood to be antigen precipitable, and SDS-PAGE analysis showed the labeled protein to be intact (Fig. 3). Therefore, the observed radiation in the blood, and probably in other organs, represents the entire IgG3-IL2 molecule and not a degradation product thereof.

The differences between the half lives of the prior IgG1-IL2 and present IgG3-IL-2 fusion proteins may be due to either the characteristics of the two isotypes or the manner in which IL-2 was connected to them. IgG3 actually has a shorter half-life than IgG1, thus rendering the results observed with the present IgG3 fusion proteins even more surprising and unexpected. However, IgG3 has other characteristics that make it a particularly advantageous isotype for fusion. IgG3 is unique among all of the IgGs in having an extended hinge region of 62 amino acids. This hinge serves as a spacer,[26] separating the Fab from the Fc with its attached IL-2. This spacing may allow the fusion protein to retain a more Ig-like conformation with its associated extended half-life.

In addition, there are differences in how the IL-2 moiety is fused to the end of C_H3 in the two molecules. In a preferred embodiment, the present IgG3-IL2 fusion protein contains a single cysteine between the last amino acid of C_H3 and the first amino acid of the mature IL-2 in each heavy

chain. It is possible that these free cysteines interact with each other or with other cysteine residues to stabilize the molecule. Alternatively, the orientation of the molecule may partially protect IL-2 so that it is less accessible to proteases.

The combined problems of the short half life and toxicity of IL-2 have made it difficult to achieve useful doses at the tumor site. IL-2 is quickly eliminated before it can reach dense and poorly vascularized tumor tissue. The tissue distribution of the present fusion protein (e.g., IgG3-IL2) shows that this molecule, with its longer half-life, is able to travel throughout the body and infiltrate distant organs. The ability of the present IgG3-IL2 to permeate organs such as the heart and lung suggests that it will reach tumor tissue in cancer patients. Anti-tumor forms (e.g., those in which the Ab moiety binds specifically to an antigen on the surface of a tumor cell or to a dansylated form thereof) of this molecule should, therefore, be able to bind and localize to tumor tissue in quantities sufficient to achieve immune activation at the site of the tumor.

When used to stimulate the proliferation of the IL-2 dependent cell line CTLL-2, IgG3-IL2 has a specific activity slightly lower than that of human recombinant IL-2 (hrIL-2). In marked contrast, when comparable unit concentrations (as defined by the standard CTLL-2 proliferation assay) are used to stimulate human peripheral blood lymphocytes (PBL), IgG3-IL2 generates significantly greater lymphokine activated killer (LAK) cell cytotoxicity than does hrIL-2.

Competition studies show that IgG3-IL2 binds the intermediate affinity form of the IL-2 receptor (IL-2R), consisting of the β and γ subunits, with an affinity slightly less than that of hrIL-2. In contrast, IgG3-IL2 shows a greater affinity than hrIL-2 for the high affinity IL-2R, consisting of α, β and γ subunits.

The exemplary IgG3-IL2 fusion protein possesses a combination of the biological properties of IgG3 and IL-2, including antigen binding, complement activation, FcγRI binding, IL-2R binding and stimulation of both proliferation and LAK activity. This combination of activities allows IgG3-IL2 to target (a) humoral and cell-mediated immune activation to the site of an antigen of interest and/or (b) an antigen to IL-2R bearing cells or organs.

Antibody-IL-2 fusion proteins may be of greater therapeutic efficacy than comparable doses of either protein injected individually or in combination. First, the presence of the antibody variable region may target the protein, allowing for higher doses of IL-2 at the location of the antigen of interest.[27] The presence of IL-2 at that site should generate a cell-mediated immune response to a broad range of antigenic epitopes not limited to the antibody's target ligand. Second, IL-2 is unstable in vivo and is quickly eliminated by the kidney due to its small size (15 kDa).[8] The resulting short half-life of IL-2 may be extended by MAb-conjugation due to both the size and stability of the MAb.[25] Third, by increasing the permeability of nearby blood vessels, an antibody-IL2 fusion protein may increase immune access to the antibody's target ligand. This has been observed with IL-2 plus MAb combination therapy and MAb-IL2 chemical conjugates.[16,28] Additionally, the simultaneous triggering of cell mediated immunity through IL-2 and Fc-mediated effector functions through the antibody could further improve immune stimulation. Finally, by simultaneously binding both antigen and IL-2 receptor (IL-2R), MAb-IL2 may cross-link IL-2R-bearing effector cells with antigen-bearing target cells or, alternatively, target some bound soluble antigen to IL-2R bearing cells.

Thus, the present invention also concerns a method of stimulating an immune response to an antigen, comprising administering the present fusion protein (i) to a patient in

need thereof (i.e., of a stimulated immune response to the antigen) or (ii) to an animal which exhibits an immune response and which has cells having a surface receptor for the Ck moiety of the fusion protein, in an amount effective to stimulate the patient's or animal's immune response. In further embodiments, the cytokine moiety is IL-2, the receptor is an IL-2 receptor, the antigen or hapten is a dansylated polypeptide, protein, or amino sugar (e.g., such as those located on a cell surface), and the antibody is an anti-dansyl monoclonal antibody.

The present method of stimulating an immune response to an antigen is useful for increasing the immune response to one or more antigens associated with one or more particular diseases (e.g., where the amino group-containing hapten is an HIV or other retroviral coat protein, a viral coat protein, an enterotoxin or other bacterial toxin, a fungal protein, an antigenic region of such a protein or toxin, an amino sugar of a bacterial or fungal cell wall, etc.). Thus, in such cases, the present method is also useful for treating a viral, bacterial, fungal or retroviral infection.

To determine if the activities of MAbs and IL-2 can be combined, a fusion protein was created linking the N-terminus of human IL-2 to the C-terminus of human IgG3. Earlier studies have shown that an IgG1-IL2 fusion protein binds antigen, generates ADCC, stimulates T cell proliferation and increases the cytotoxicity of various cell types.[12,13,14,29]

In the present invention, the exemplary IgG3-IL2 is shown to be similar to IgG3 in its *N*-glycosylation and its ability to activate complement to lyse antigen coated cells. Further, IgG3-IL2 binds FcγRI with an affinity only slightly lower than that of IgG3. IgG3-IL-2, like IL-2, is able to sustain CTLL-2 proliferation and generate LAK cells from human PBL. Surprisingly, similar unit concentrations of hrIL-2 and IgG3-IL2, as determined by the standard CTLL-2 proliferation assay,

generate different levels of LAK cell cytotoxicity, with IgG3-IL2 stimulating significantly more cytotoxicity than hrIL-2.

Finally, the exemplary IgG3-IL2 binds the intermediate affinity IL-2 receptor (IL-2R$\beta\gamma$) with an affinity slightly lower than that of hrIL-2, but shows significantly higher affinity than hrIL-2 for the high affinity IL-2 receptor (IL-2R$\alpha\beta\gamma$). The discrepancy between proliferative activity and LAK generation of IgG3-IL2 may be viewed in light of the affinity for the receptor forms and known functions of the receptor subunits.

IgG3-IL2, an exemplary fusion protein designed to combine the antigen specificity and effector functions of human IgG3 with the immune stimulatory activities of IL-2, has been engineered and expressed. By fusing these two molecules, a therapeutic agent with an improved repertoire of properties and activities has been provided.

Prior studies have been extended by the present invention to show that the exemplary IgG3-IL2 is *N*-glycosylated, activates complement, binds FcγRI, binds both intermediate and high affinity IL-2Rs, and generates LAK cell cytotoxicity. In two respects (generation of LAK cell cytotoxicity and affinity for the holoreceptor IL-2R($\alpha\beta\gamma$)), IgG3-IL2 has significantly improved properties compared to IL-2.

Production of IgG3-IL2 may require that the heavy chain gene fusion be transcribed, translated, assembled with its light chain and secreted by transfected cells. Fig. 1 demonstrates that the exemplary IgG3-IL2 is properly assembled and secreted as the expected 200-kDa H_2L_2, disulfide-linked heterotetramer and that it retains its ability to bind antigen.

Thus, the present invention further concerns a method of producing a fusion protein, comprising:

culturing a cell line transformed with a gene encoding a fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody, L is a covalent bond or naturally-occurring amino acid-containing linking group, and Ck is a known cytokine, lymphokine or biologically active fragment thereof, in a medium supporting the growth of the cell line for a length of time sufficient to express the fusion protein, and

recovering the fusion protein from the cell culture.

In preferred embodiments, the antibody comprises one or more κ light chains and/or heavy chains (more preferably two of each), which may be expressed from the same or different polynucleotide strands; the linking group L is a cysteine residue; and the cytokine is IL-2 (preferably human IL-2). The linking group may join the C-terminus of the C_H3 chain of the antibody to the N-terminus of the cytokine.

The cell line is preferably one capable of expressing the polynucleotide(s) and permitting assembly of antibody chains, subunits and/or fragments (where necessary) without substantial proteolytic degradation of the fusion protein.

The present fusion protein may be recovered by precipitating from the culture supernatant with dansyl-BSA-coated beads, or other affinity purification techniques. Cultured cells may be lysed or ruptured prior to recovering the fusion protein.

The Fc region of IgG3, and its *N*-glycosylation site, are known to be involved in both Fc receptor binding and complement activation.[30,31] Therefore, the utilization of this glycosylation site in the fusion protein is important to the effector functions of the molecule. The shift in molecular weight of the protein produced in the presence of tunicamycin indicates that this site is glycosylated in IgG3-IL2 (Fig. 1A, lane 2 and Fig. 1B, lane 2). Thus, the gene fusion is transcribed, translated, post-translationally modified, assembled and secreted as desired.

Each chain of the Fc region of IgG3, with its complement activation and FcγRI (CD64) binding domains, is fused to a 15-kDa IL-2 molecule in IgG3-IL2. While previous reports have shown that an IgG1-IL2 fusion protein mediates ADCC in IL-2 stimulated LAK cells and NK cells which express the low affinity FcγRIII (CD16) and IL-2R,[13,29] no studies have examined complement activation and FcγRI binding by the fusion proteins. Here, it is shown that IgG3-IL2 binds to the high affinity FcγRI with an affinity only slightly less than that of IgG3. It has been further shown that IgG3-IL2 is able to activate complement to lyse antigen coated cells (Fig. 3). Activation of complement leads to lysis of antigen-bearing cells and release of chemotactic factors. Together, Figs. 2 and 3 demonstrate that the Fc region of IgG3-IL2 molecules, retains its appropriate structure and effector functions.

Retention of IL-2 activity is a critical property of antibody fusion proteins comprising IL-2. The exemplary IgG3-IL2 differs in two significant ways from the previously described fusion proteins: (1) human IgG3 is used instead of IgG1 for the fusion, and (2) the present IgG3-IL2 fusion protein does not have a linker separating IL-2 from the end of CH3, but instead, has a cysteine residue at the joint.

The present IgG3-IL2 fusion protein is of intermediate cytokine activity, being 30-60% as potent as IL-2 in stimulating CTLL-2 proliferation (Fig. 4), but more potent than prior IgG1-IL2 fusion proteins. Therefore, variations in the Ab "partners" used for IL-2 fusion may influence the activity of the resulting protein.

The therapeutic value of IL-2 lies in its ability to stimulate an immune response, including generating increased cytotoxic activity from a variety of immune cells. The generation of LAK cells from PBL is a property of IL-2 that has been well studied and applied to the treatment of human cancer.[1,4,32] The ability of the IgG1-IL2 fusion protein to direct the killing of LAK cells generated with IL-2 was

previously reported.[13] However, the ability of this fusion protein itself to generate LAK cells was not examined.

In the present invention, IgG3-IL2 was found to stimulate more cytotoxicity than hrIL-2 at every effector to target ratio, even when hrIL-2 was added at a 10-fold higher unit/ml concentration (Fig. 5). Thus, hrIL-2 and IgG3-IL2 differ significantly in their relative ability to generate (1) proliferative activity in the CTLL-2 assay and (2) cytotoxicity in the LAK assay. This difference may reflect the stability of the antibody molecule, which may enable the fusion protein to remain active for a longer period of time during the 3-to 5-day culture involved in the LAK assay. Alternatively, the bivalency of IgG3-IL2 may affect the signaling of the molecule or the internalization and degradation process that eventually leads to cessation of signaling. The bivalent nature of IgG3-IL2 may also lead to an increased overall affinity for its membrane bound receptor, leading to greater receptor occupancy and greater signaling.

To determine if the discrepancy between proliferative and cytotoxic activities was reflected in differences in IL-2R binding, the ability of hrIL-2 and IgG3-IL2 to compete with ^{125}I-labeled IL-2 for binding to the IL-2R was analyzed. IgG3-IL2 exhibits a slightly lower affinity than hrIL-2 for the intermediate affinity IL-2R($\beta\gamma$) (Fig. 6A). In marked contrast, IgG3-IL2 shows a considerably higher affinity than hrIL-2 for the high affinity IL-2R($\alpha\beta\gamma$) (Fig. 6B). Similarly, using ^{125}I-labeled IgG3-IL2, IgG3-IL2 exhibited higher affinity than hrIL2 for the high affinity IL-2R($\alpha\beta\gamma$) (Fig. 6C).

Several characteristics of IgG3-IL2 could impact upon its differential binding to these two forms of the IL-2R subunits.[33] The fusion of an antibody moiety (e.g., IgG3) to IL-2 may alter the structure of IL-2 or sterically hinder it such that selected portions of IL-2 are less accessible for binding. Additionally, the size and structure of IgG3-IL2 may impair its binding to multiple, large IL-2R ($\beta\gamma$) dimers,

making it effectively monovalent for this intermediate affinity receptor, while the smaller size of the IL-2R alpha subunit (p55, TAC), or its interaction with a different surface of IL-2, could allow a bivalent interaction with the two IL-2 moieties on the fusion protein. Alternately, the fast association time of the alpha subunit may speed the initial contact between the cell surface and IgG3-IL2, allowing the bivalent IgG3-IL2 molecule to then bind the intermediate affinity subunit, with its slower dissociation rate, more efficiently.

The characteristics of the present exemplary IgG3-IL2 fusion protein suggest multiple applications for this molecule. The dansyl specificity of this protein could potentially be used to deliver any dansylated molecule to IL-2R bearing cells or organs. Candidate molecules include toxins which may be delivered specifically to IL-2R bearing cells for the therapy of virally infected T-cells, T-cell leukemias or autoimmune diseases.

Thus, the present method of stimulating an immune response to an antigen may further comprise the step of dansylating a target antigen prior to administering the same (either separately from or in combination with an anti-dansyl Ab-L-Ck fusion protein) to the animal or patient.

The improved binding to the high affinity form of the IL-2R may increase the specificity of IgG3-IL2 for various T cell types. Alternatively, targeting of dansylated antigens to IL-2R bearing cells may affect the overall immune response to that antigen by altering the cells that encounter and ultimately present the antigen, or by affecting the pathway of endocytosis or activated status of the antigen presenting cell itself.

The high affinity of IgG3-IL2 for IL-2R($\alpha\beta\gamma$) may make it efficient in imaging IL-2R($\alpha\beta\gamma$) bearing tumors (although it is expected to be effective in imaging any IL-2 receptor-bearing cell or tissue). In this case, the fusion protein may be

labeled, for example with a radioactive isotope (e.g., ^{3}H, ^{35}S, ^{32}P, ^{125}I, ^{51}Cr, etc.), a fluorescent label (e.g., dansyl, biotin, eosin, rhodamine, a flavinoid such as FAD, or other chromophore such as those available from Molecular Probes, Inc.), or other means for detecting the target (e.g., tissue, organ or cell) to which the labeled fusion protein is bound.

Additionally, by changing the antigen binding specificity of the IgG3-IL2 fusion proteins, IL-2 activity can be selectively targeted to regions containing the recognized antigen. Tumor specific fusion proteins have been shown to suppress the growth of metastases in a human LAK cell reconstituted scid mouse model.[13]

The combination of antibody and IL-2 in a fusion protein creates a versatile molecule with a number of useful properties. Successful application of this molecule may depend on a clearer understanding of its full range of activities. It is noteworthy that different antibody-IL2 fusion proteins have somewhat different characteristics.[12,13,34] The present IgG3-IL2 fusion protein activates complement, binds antigen, FcγRI and IL-2R and stimulates both proliferation and cytotoxicity. The noted increases in LAK generation and binding to the high affinity IL-2R($\alpha\beta\gamma$) are unexpected findings that highlight the importance of the present fusion protein.

The ultimate utility of antibody-IL2 fusion molecules depends on their pharmacological properties. In addition to its toxicity, IL-2 has a very short half-life *in vivo*, necessitating difficult regimens of continuous infusion or multiple daily injections to maintain effective, yet subtoxic blood concentrations. Antibodies, with their *in vivo* half-lives of days, could be expected to contribute to an extended half-life for an antibody-IL2 fusion protein by increasing the size and stability of the molecule.

In the present invention, an Ab-cytokine (preferably IgG3-IL-2) fusion protein (IgG3-IL2) injected into the

peritoneum of mice demonstrates a half-life of at least 1 hour, preferably at least 2 hours, more preferably at least 4 hours and most preferably at least 7 hours, much longer than that of IL-2[8] or of the previously described fusion between IgG1 and IL-2.[35]

The present Ab-cytokine fusion protein (preferably IgG3-IL2) escapes the peritoneal cavity and permeates organs throughout the injected animal, including the heart and lungs, and tends to localize to organs bearing cytokine receptors (e.g., IL-2R), especially the thymus. The fusion protein is recoverable from the blood of mice four hours after intraperitoneal injection, and SDS-PAGE analysis of the recovered protein indicates that it is intact. Mice are well able to tolerate injections of 100 μg of Ab-cytokine (e.g., IgG3-IL2), indicating that the increased half-life does not result in dramatically increased toxicity.[35,36]

In a more specific embodiment, the present inventors show herein that the mouse antibody response to an injected antigen is enhanced when that antigen is bound by IgG3-IL2, but not IgG3. Increases were noted, especially at early time points, in the production of antigen-specific antibodies of all measured isotypes: IgA, IgM, IgG1, IgG2 and IgG3. The IgG2 response was particularly dramatic, in agreement with previous reports that IL-2 increases secretion of this isotype.[37]

In a preferred embodiment, the antibody is a monoclonal antibody of the IgG2, IgG3, IgA or IgM isotype. Preferably, the antibody moiety is a monoclonal antibody that specifically binds to a dansyl hapten. The hapten group dansyl is very reactive and can be easily attached by known methods to virtually any protein.

When an anti-dansyl/IL-2 fusion protein is injected along with a dansylated antigen, there is an greatly increased antibody response to the antigen. Presumably, the anti-dansyl/IL-2 fusion protein remains localized at the site of the antigen, triggering a more potent immune response.

A similar approach can be used to potentiate the immune response to a large number of different antigens. Potential uses of the present fusion protein include:

(a) injecting it along with antigen into mice and producing hybridomas with the mice;

(b) injecting it with antigen into other animals (preferably mammals, more preferably rabbits and/or goats) to elicit a more potent immune response;

(c) attaching dansyl to the surface of a cell such as a tumor cell, and injecting the dansylated tumor cell coated with anti-dansyl/IL-2 into a host animal or patient to elicit a more potent anti-tumor response;

(d) attaching dansyl to the surface of an infectious agent such as a bacterium or a virus and injecting the infectious agent along with anti-dansyl/IL-2 to elicit a more potent specific immune response; and

(e) attaching dansyl to a toxin to target that toxin to IL-2 receptor-bearing cells and administering the same to a patient in need thereof to treat T cell leukemias, an autoimmune condition or prevent rejection of a transplant.

The above approaches may be especially useful in situations where the patient is immunologically compromised.

Mice models are shown hereinbelow to tolerate doses of 100 μg of the present IgG3-IL2, although with some weight gain. This dose of over 7,000 units/g is 70 times greater than the dose of hrIL-2 that causes similar weight gain in humans, indicating that IgG3-IL2, with its extended half-life *in vivo*, is not proportionally increased in toxicity.[17]

A major objective of the present invention is to determine if the immune stimulatory activities of a cytokine (e.g., IL-2) could be coupled with the antigenic specificity of an antibody. One application of the present fusion protein is thus to target a cytokine (e.g., IL-2) to the site of a solid tumor, where it may stimulate an anti-tumor immune

response. Indeed, antibody-IL-2 fusion proteins have utility in generating an anti-tumor immune response (see, e.g., the present disclosure and ref. 9 hereinbelow).

A further application of the present IgG3-IL2 fusion protein is in the production of vaccines against molecules attached to IL-2 via the antigenic specificity of the antibody. Direct fusion of IL-2 to candidate antigens has shown that an increased immune response is generated against those antigens when they are inoculated into animals as fusion proteins with IL-2.

An antibody-IL2 fusion protein was able to generate immunity to the B cell lymphoma that expressed that same antibody on its surface.[19] Fusion to IL-2 increased the immunogenicity of a herpes simplex virus (HSV) glycoprotein and provided greater protection against challenge with HSV than when protein adsorbed to alum as an adjuvant was used.[20-23] While these experiments have demonstrated that IL-2 fusions are useful as vaccines, to date this approach has required the tedious and time consuming process of fusing molecules at the gene level, expressing and purifying the fusion gene product, which may or may not retain IL-2 activity.

The present anti-dansyl IgG3-IL2 fusion protein provides a tool which can be used to join bioactive IL-2 to any protein of interest to potentiate the immune response against the associated protein. The hapten dansyl can be easily linked to any primary amine present on a potential antigen, and the resulting dansylated antigen is then recognized by the high affinity IgG3-IL2. The antigen-antibody complexes can then be inoculated into animals, and the resulting immune response may provide protection against challenge with antigen bearing virus, bacteria or tumor cells.

Using BSA as a model antigen, the results indicate that the antibody response is greatly enhanced by the attached fusion protein IgG3-IL2, but not by attached Ab (IgG3) alone.

Antigen coated beads generate an antibody response that

is changed only slightly when the beads are bound by human IgG3 before injection. However, when bound by the present fusion protein (IgG3-IL2), the increase in antibody response is dramatic. All isotypes are increased starting early after injection and remain elevated compared to controls for the length of the study. This early response may reflect the ability of the present fusion protein (IgG3-IL2) to directly stimulate antibody producing cells, relieving the need for T helper cells for activation. Alternatively, the local concentration of IL-2 may stimulate other nearby cells to produce cytokines that then act on the B cells to stimulate antibody production.

The particularly dramatic increase in IgG2 caused by the present fusion protein (e.g., IgG3-IL2) is consistent with the earlier observation that IL-2 stimulates increased IgG2a production *in vitro*.[36] Thus, present *in vivo* results confirm earlier predictions based on *in vitro* results. Ab (IgG3) alone had only minimal effect on the antibody response, with notable increases in IgG1 response and the IgM response to boost.

In this preferred embodiment, the effects of IgG3 do not resemble those of IgG3-IL2 in either scale or pattern. Therefore, the majority of the effects of IgG3-IL2 on the antibody response to antigen may be attributed to the IL-2 portion of this molecule.

The large increase in antibody production in mice immunized with a complex of the present fusion protein (e.g., IgG3-IL2) bound to antigen indicates that this approach to vaccination may be successful with other antigens. Of particular interest is the ability of this type of molecule to stimulate an immune response in immune compromised individuals, such as AIDS patients, who may lack effective cytokine secreting T helper cells. IL-2 may improve the immune status of such patients.[39] The extended half-life of the present fusion protein (e.g., IgG3-IL2) and its ability to

attach to an antigen of interest may greatly improve upon the ability of IL-2 to bolster the immunity of such patients.

The extended half-life and versatility of the present fusion protein (e.g., IgG3-IL2) provides a novel tool for both scientists and clinicians to use targeted IL-2 as an immune stimulator. In addition to the anti-tumor applications, the properties of the present fusion protein (e.g., IgG3-IL2) suggest it may be useful in other areas, such as in treatment of AIDS and of secondary opportunistic infections associated therewith. The long half-life of this protein may allow immune compromised patients to be treated with relatively constant blood concentrations of IL-2, effectively boosting their immune system. Additionally, the longer half-life of the present fusion protein may result in a reduced frequency of IL-2 administration to AIDS patients, reducing thus the risks of exposure of health care professionals to HIV-infected blood. Finally, as a versatile protein able to recognize a panel of dansylated antigens, anti-dansyl IgG3-IL2 may facilitate producing vaccines using IL-2 associated with many different antigens.

The increased half-life and immune stimulatory activities of the present fusion protein (preferably IgG3-IL2), particularly when coupled with the versatility of the dansyl system, provide a novel tool for the generation of cytokine-(e.g., IL-2)-potentiated immune responses.

Other features of the present invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention, and are not intended to be limiting thereof.

EXAMPLES

Experiment 1: Production and characterization of an Ab-IL2 fusion protein

Materials and methods

1. Plasmid construction

RNA from PMA and PHA stimulated Jurkat cells was used to generate cDNA by reverse transcription using an oligo dT primer. This cDNA was used as template for a polymerase chain reaction (PCR) using oligonucleotide primers flanking the mature IL-2 coding sequence: primer 1, 5'-ACAACACGTG CACCTACTTC AAGTTC-3'; primer 2, 5'-GCACTGAATT CTCAAGTCAG TGTTGA-3'. The PCR product was cloned into pBluescript and sequenced. Using the PmlI and *Eco*RI sites introduced during the PCR reaction, the IL-2 cDNA was subcloned into a vector created to allow fusion of various genes to the human IgG3 heavy chain at the 3' end of the IgG3 constant domain (C_H3).[39] This vector contains a *Pvu*II site which yields a blunt end two base pairs after the end of C_H3. The IL-2 cDNA fragment digested with PmlI results in a single base pair before the first codon of mature IL-2. By ligating these fragments, the resulting fused genes encode a fusion protein linking the last amino acid of C_H3 to the first amino acid of mature IL-2 with a single codon, generated as a consequence of the cloning strategy, encoding a cysteine between them. The fused gene was then joined to a mouse anti-dansyl (dansyl: 5-dimethylaminonaphthalene 1-sulfonyl chloride) variable region in the expression vector $pSV_2\Delta Hgpt$.[38] The expression vector encoding the chimeric mouse/human kappa (κ) light chain specific for DNS ($pSV_{1\kappa}\Delta HneoV_{DNS}Huk$) is available from the present inventors.

2. Protein expression and characterization

The IgG3-IL2 fusion heavy chain and κ light chain expression vectors were transfected into P3X63Ag8.653 by electroporation.[40] Transfectomas were selected with G418

(Gibco, Grand Island, NY) at 1.0 mg/ml and screened for production of IgG3-IL2 by an enzyme-linked immunosorbent assay (ELISA). A clone shown to secrete IgG3-IL2 at 0.2 $\mu g/10^6$ cells/ml/24 h was selected and named TAAG. ^{35}S-labeled IgG3-IL was prepared by growing TAAG cells, in the presence or absence of tunicamycin, in DMEM minus methionine (Gibco, Grand Island, NY) supplemented with [^{35}S]methionine (ICN, Irvine, CA) and precipitating the protein from the supernatant with dansyl-BSA coated Sepharose.[39] Unlabeled IgG3-IL2 was purified from culture supernatants using an affinity column with the dansyl isomer 2-dimethylamino-naphthalene-5-sulfonyl chloride (Molecular Probes, Eugene, OR) coupled to AH-Sepharose 4B. Bound protein was eluted with the dansyl isomer *N*-5-carboxypentyl-2-dimethylaminonaphthalene-5-sulfonamide (Molecular Probes, Eugene, OR) and hapten removed by extensive dialysis.[41] Purity was assessed by Coomasie blue staining of SDS-polyacrylamide gels and determined to be greater than 95% for proteins used in these experiments. Highly purified hrIL-2 was kindly provided by Cetus Corporation (Emeryville, CA). The specific activity of the hrIL-2 was 1.8×10^7 International Units (IU)/mg of protein. Protein concentration was determined by a bicinchoninic acid based protein assay (BCA Protein Assay, Pierce Chemical Co., Rockford, IL). Proteins analyzed by SDS-PAGE were visualized by exposure on a phosphor screen with subsequent analysis on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

3. *Cell Lines*

YT-1, a human natural killer-like cell line that expresses the intermediate affinity IL-2R receptor,[12] was kindly provided by Warner Greene (UCSF, San Francisco, CA). The human histiocytic lymphoma cell line U937 that expresses the FcγRI receptor[43] was kindly provided by Leonard Chess (Columbia University, NY). CTLL-2, an IL-2 dependent murine T cell line, and Raji, an NK-resistant human Burkitt's lymphoma,

were kindly provided by William Clark (UCLA, CA). YT-1, U937 and Raji were grown in RPMI 1640 medium supplemented with 10% (vol/vol) calf serum (HyClone, Logan, UT). YT-1 cells were induced to express the high affinity IL-2R by stimulation with 10 μM forskolin (Sigma Chemical Co., St. Louis, MO) for 24 h at 3-5 x 10^5 cells/ml (designated YT-1*). U937 cells were stimulated with interferon-γ (100 units/ml) for 2 days before binding assays to increase FcγRI expression. P3X63Ag8.653 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) with 5% calf serum (HyClone, Logan, UT). CTLL-2 cells were cultured in RPMI 1640 medium supplemented with 10% bovine calf serum and IL-2.

4. Receptor binding assays

IgG3 and IgG3-IL2 were radiolabeled with Na-^{125}I (Amersham, Arlington Heights, IL) using Iodobeads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol. ^{125}I-labeled IL-2 was purchased from New England Nuclear (Wilmington, DE). For binding assays YT-1, YT-1* or U937 cells were washed twice with RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum (CM) and resuspended at 2-4 x 10^7 cells/ml in CM. Aliquots of this cell suspension (90 μl) were then added to 10 μl of CM containing radiolabeled ligand and cold competitor at varying concentrations in 0.5-ml tubes. These samples were rotated for 3 h at 4°C for IL-2R assays and at 13°C for FcγRI assays. Bound and free iodinated protein were separated by layering the suspension over 100 μl binding oil (84% paraffin oil, 16% silicon oil) and spinning for 1 min in a microcentrifuge. After freezing at -70°C, the bottom of each tube was cut off and the radioactivity of the cell pellet and medium were measured in a Beckman Gamma 5500 counter (Beckman Instruments, Fullerton, CA).

5. Complement-mediated hemolysis

Sheep red blood cells (SRBC) were coated with DNS-BSA by incubation at 30°C for 1 h in 150 mM NaCl, 0.25 mM $CrCl_3$, pH 7.0 with 0.25 mg/ml DNS-BSA. Then, 0.2 ml packed, antigen coated SRBC were incubated for 1 h at 37°C with [^{51}Cr]sodium chromate (Amersham Corp., Arlington Heights, IL) in 2 ml of fresh Gel-HBS buffer (0.01 M Hepes, 0.15 M NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.1% gelatin, pH 7.4). The free [^{51}Cr]sodium chromate was removed by washing the cells three times in 10 ml of fresh Gel-HBS. Then 50 µl of 2% ^{51}Cr-loaded, DNS-BSA coated SRBC in Gel-HBS were added to round bottomed, 96-well plates (Corning Glass Works, Corning, NY) containing 50 µl of antibodies at various concentrations in Gel-HBS. Ten CH_{50} units of human serum, preabsorbed against unlabeled SRBC, in a volume of 25 µl were added to each well sequentially. The plates were incubated at 37°C for 45 min, unlysed SRBC were pelleted by centrifugation of the plate and 50 µl of supernatant removed and counted in a gamma counter. Each point was assayed in duplicate and the percentage lysis calculated.

6. *Proliferation*

A 96-well plate containing serial dilutions of hrIL-2 or IgG3-IL2 in 50 µl CM was seeded with 50 µl CM containing 2-4 x 10^4 CTLL-2 cells previously depleted of IL-2 for 4 h. After incubation for 18 h, 1 µCi [methyl-^{3}H]thymidine in 24 µl CM was added to each well and incubated for 6 h longer. The cells were then harvested from the wells with dH_2O and washed through glass microfiber filters (Whatman Ltd., Maidstone, UK). The filters were then immersed in EcoLume (Amersham, Arlington Heights, IL) and quantitated in a Beckman LS3150T liquid scintillation counter (Beckman Instruments, Fullerton, CA).

7. *Lymphokine activated killer (LAK) assay*

LAK cell activity was determined as previously described.[4] Briefly, PBMC were isolated from human blood using a ficoll

gradient and depleted of monocytes and macrophages by adherence to plastic tissue culture plates twice for 1 h. The non-adherent cells were cultured with various concentrations of hrIL-2 or IgG3-IL2 in CM for 3-5 days, harvested, washed with CM and used as effectors in a standard 4-h ^{51}Cr release assay as previously described.[44] Raji cells were used as targets. Percentage specific lysis was calculated as the fraction [(experimental release - background release)/(Triton release - background release)]/100. Experiments were performed in triplicate and error bars represent the standard deviation. Background release was defined as that in the presence of an equal number of unstimulated effectors.

Results:

1. *IgG3-IL2 is assembled and secreted as H_2L_2*

IgG3 is composed of two heavy chains (H) and two light chains (L) assembled and secreted as a disulfide-linked heterotetramer (H_2L_2). To determine if IgG3-IL2 is similarly assembled and secreted as H_2L_2, cells producing IgG3-IL2 (TAAG) were biosynthetically labeled by growth in [^{35}S]methionine and IgG3-IL2 was precipitated from the cell supernatant using dansyl-BSA coated Sepharose. Precipitated protein was examined by SDS-PAGE in the absence (Fig. 1A, lane 3) and presence (Fig. 1B, lane 3) of reducing agent to determine the molecular weight of the assembled protein and its constituent subunits, respectively.

The intact protein migrates slightly faster than a 200-kDa molecular weight standard. Upon treatment with β-mercaptoethanol, it is reduced to subunits of 28 kDa, corresponding to the light chain (L), and 70 kDa, the expected molecular weight for the IgG3 heavy chain (H) plus IL-2. Therefore, H and L chains of the appropriate size are synthesized, assembled and secreted as the expected H_2L_2 heterotetramer. Treatment of cells with tunicamycin, an inhibitor of *N*-glycosylation, causes a decrease in the

apparent molecular weight of the heavy chain (Fig. 1A, lane 2 and Fig. 1B, lane 2), indicating that the sole recognition site for N-glycosylation within the C_H2 domain of IgG3 is utilized in the fusion protein.

2. IgG3-IL2 binds FcγRI and activates complement

IgG3-IL2 contains the Fc region of IgG3 and should possess many of the Fc-associated effector functions. To assess the ability of IgG3-IL2 to bind to the human high affinity Fc receptor, FcγRI, competition studies were performed using U937 cells stimulated with gamma interferon to express approximately 20,000 FcγRI per cell (ref. 42 and data not shown). ^{125}I-labeled IgG3 binding was measured in the presence of various concentrations of IgG3 or IgG3-IL2 as unlabeled competitors (Fig. 2).

Both competitors inhibited binding in a dose-dependent manner and were capable of 100% competition. Half maximal inhibition of binding was obtained with 1-2 nM IgG3 or 3-4 nM IgG3-IL2, indicating that IgG3-IL2 binds FcγRI with slightly decreased affinity compared to IgG3. Neither IgG2 nor HrIL2 affected binding in these assays. Similar results were obtained using ^{125}I-labeled IgG3-IL2 as the labeled ligand (data not shown).

In order to determine whether IgG3-IL2 interacts with the complement system, the ability of IgG3-IL2 to activate complement through the classical pathway to bring about lysis of antigen coated sheep red blood cells was evaluated. IgG3-IL2 was able to direct lysis of these cells in a dose-dependent manner (Fig. 3), although the lysis achieved by IgG3-IL2 was somewhat less than that of IgG3 at similar concentrations. Neither IgG3 or IgG3-IL2 lysed SRBC in the absence of complement (data not shown).

3. IgG3-IL2 stimulates both proliferation and cytotoxicity

In order to determine if the IL-2 portion of the protein is functional, IgG3-IL2 was tested for its ability to stimulate proliferation of the IL-2 dependent murine cell line CTLL-2. Proliferation was assessed by measuring the incorporation of [^{3}H]-thymidine into newly synthesized DNA. Maximal proliferation levels achieved with IgG3-IL2 were at least as high as those achieved with hrIL-2 (Fig. 4). Half maximal proliferation was achieved with IgG3-IL2 concentrations of 0.2-0.4 nM, slightly higher than the 0.1-0.2 nM of hrIL-2 required to achieve half-maximal proliferation. Moreover, hrIL-2 stimulated some proliferation at concentrations as low as 10-20 pM, whereas 80-100 pM of IgG3-IL2 were required to stimulate similar low level proliferation. Both hrIL-2 and IgG3-IL2 activities in this assay were abrogated by anti-IL-2 neutralizing antibodies (data not shown). Similar specific activities were observed in repeated assays with multiple preparations of IgG3-IL2 using CTLL-2 or the IL-2-dependent murine cell line HT-2 (data not shown).

To compare the relative ability of IL-2 and IgG3-IL2 to generate LAK cell activity human PBLs incubated for 3-5 days with hrIL-2 or IgG3-IL2 at various concentrations were assayed for their ability to lyse ^{51}Cr-labeled Raji target cells. The two proteins were compared at equivalent IU concentrations based on the CTLL-2 proliferation assay; therefore, the molar concentration of IL-2 was 30% of that of IgG3-IL2 at the same IU/ml concentration.

Both hrIL-2 and IgG3-IL2 increased killing in a dose-dependent manner (Fig. 5). Maximal killing (defined as greater than 95% ^{51}Cr release) was generated at 1000 IU/ml for hrIL-2 and an effector to target (E/T) ratio of 50. In contrast, IgG3-IL-2 required only 100 units/ml for maximal killing at an E/T of 50. It is noteworthy that IgG3-IL2 consistently generated more efficient killing than a 10-fold higher concentration of hrIL-2 at all E/T ratios.

Furthermore, significant activity is generated at IgG3-IL2 concentrations of 10 IU/ml, a concentration at which very little activity was observed with IL-2 at any E/T ratio. The data is representative of numerous similar experiments.

4. IgG3-IL2 binds both intermediate and high affinity IL-2 receptors

To assess binding to IL-2R, competition studies were performed using the human cell line YT-1 and ^{125}I-labeled IL-2 with varying concentrations of unlabeled hrIL-2 or IgG3-IL2. YT-1 cells express approximately 30,000 intermediate affinity (K_d = 0.5-1.0 nM) IL-2 receptors (IL-2R$\beta\gamma$) on their surface (ref. 41 and data not shown). Both hrIL-2 and IgG3-IL2 inhibited binding of ^{125}I-labeled IL-2 in a dose-dependent manner and to the same maximal levels (Fig. 6A). Half maximal inhibition occurred at 3-4 nM for hrIL-2 and at 7-8 nM for IgG3-IL2, suggesting a slightly lower affinity of the fusion protein for IL-2R($\beta\gamma$).

To examine binding to the high affinity IL-2 receptor (IL-2R$\alpha\beta\gamma$), expression of the alpha subunit was induced in YT-1 cells (designated YT-1$^+$) by 24-h incubation with 10 μM forskolin. This treatments results in the surface expression of approximately 400 high affinity IL-2R($\alpha\beta\gamma$) per cell.[41] Both hrIL-2 and IgG3-IL2 inhibited binding of ^{125}I-labeled IL-2 to these cells in a dose-dependent manner and to similar maximal values (Fig. 6B). However, half maximal inhibition occurred for hrIL-2 at 2-3 nM, while IgG3-IL2 required only 0.7 - 0.8 nM, indicating that, in contrast to what was observed with the intermediate affinity IL-2 receptor, IgG3-IL2 has a significantly higher affinity than hrIL-2 for the high affinity form of the IL-2 receptor.

To confirm this result, ^{125}I-labeled IgG3-IL2 binding to YT-1$^+$ cells in the presence of varying concentrations of unlabeled hrIL-2 or IgG3-IL2 was examined (Fig. 6C). Both hrIL-2 and IgG3-IL2 inhibited binding of ^{125}I-labeled IgG3-IL2

to these cells in a dose-dependent manner and to similar maximal values. Half maximal inhibition occurred for hrIL-2 at 10-15 nM and for IgG3-IL2 at 0.9-1 nM, again indicating that IgG3-IL2 has a higher affinity than hrIL-2 for this form of the IL-2 receptor. Unlabeled IgG3 did not affect binding to IL-2R in these assays (data not shown).

Experiment 2: Characterization of Biological Activity and Potentiation of Immune Response Using the Present IgG3-IL2 Fusion Protein

Materials and methods

Protein Purification and Characterization

Chimeric IgG3 and IgG3-IL2 contain mouse variable regions specific for dansyl (5-dimethylaminonaphthalene-1-sulfonyl chloride) and human constant regions of IgG3, kappa light chain and human IL-2. Expression and affinity purification of these proteins was performed according to Experiment 1 above. Purity was assessed by Coomasie blue staining of SDS-polyacrylamide gels. Protein concentration was determined by a bicinchoninic acid-based protein assay (BCA Protein Assay, Pierce Chemical Co., Rockford, IL). IgG3 and IgG3-IL2 were radiolabeled with Na-^{125}I (Amersham, Arlington Heights, IL) using Iodobeads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol. Proteins analyzed by SDS-PAGE were visualized by exposure on a phosphor screen and analyzed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Half-life

^{125}I-labeled IgG3 and ^{125}I-labeled IgG3-IL2 were injected intraperitoneally into adult female BALB/c mice (Taconic Farm, Germantown, NY). A whole body gamma counter (Wm. B. Johnson & Assoc. Inc., Montville, NJ) was used to determine the residual radioactivity. The percentages expressed in Figure 1 were determined by comparing the residual counts per minute to the counts per minute immediately after injection.

Biodistribution and toxicity

Female BALB/c mice were treated with KI and injected with ^{125}I-labeled protein as described above. Four hours after injection, mice were sacrificed and dissected to determine the tissue distribution of the injected protein. Organs were weighed on a Mettler H30 analytical balance (Mettler, Hightstown, NJ), and residual radiation was quantitated using the whole body gamma counter. The radiolocalization values expressed in Figure 2 were determined by comparing the counts per minute per gram of each organ with that of the entire mouse using the formula: % = [(cpm of tissue/g of tissue)/(cpm of total body/g of total body)] x 100. For toxicity studies, mice were injected intraperitoneally with unlabeled protein and observed for morbidity and weight change over time.

Blood recovery

Blood was collected from mice sacrificed 4 hours after intraperitoneal injection of ^{125}I-labeled protein. The blood was allowed to clot for one hour at room temperature, spun in a microcentrifuge and the serum transferred to fresh tubes. Thirty µl DNS-BSA-Sepharose was added to the serum and the suspension rotated overnight at 4°C. The protein bound to the antigen-coated Sepharose was recovered by spinning, washing the pellet with PBS and resuspending in sample buffer containing SDS. The samples were analyzed by SDS-PAGE under non-reducing and reducing conditions and were visualized by exposure on a phosphor screen with subsequent analysis on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Immunization with IgG3-IL2 coated DNS-BSA-Sepharose

Dansyl was conjugated to bovine serum albumin (BSA) at a ratio of 40 to 1 to make dansyl-BSA (DNS-BSA), which was used to coat Sepharose as previously described.[18] Female BALB/c mice (five mice per group) were inoculated by intraperitoneal injection with 0.5 ml of PBS containing 0.2 ml of packed DNS-

BSA-Sepharose, alone or prebound to 10 μg of IgG3 or 10 μg of IgG3-IL2. Blood was collected in microfuge tubes by nicking the tail on the indicated days, and cells removed from serum by brief centrifugation in a microfuge. Blood was pooled from the five animals of each group and diluted 1 to 1000 (IgA, IgM, IgG2, IgG3, Kappa light chain) or 1 to 5000 (IgG1) in PBS with 1% gelatin for analysis. ELISAs were performed in 96-well plates (Corning Glass Works, Corning, NY) coated with DNS-BSA at a concentration of 100 μg/ml and blocked with 1% BSA in PBS. Serum diluted in PBS was allowed to bind to the plate at 4°C overnight. The plate was washed with PBS six times and incubated with the indicated isotype-specific, alkaline phosphatase-linked antibody for 1 hour at 37°C, then washed again. Substrate (P-nitrophenyl phosphate, Sigma Chemical Corp., St. Louis, MO) was added at 0.6 mg/ml in buffer (9.6% diethanolamine, 0.24 mM $MgCl_2$, pH 9.8) for 30-60 minutes, and the absorbance (410 nm) was read using a Dynatech MR700 Plate Reader (Dynatech Labs, Inc., Chantilly, VA).

Results

The half-life of IgG3-IL2

The small size of IL-2 is believed to result in its rapid elimination from the blood by the kidney. IgG3 is a larger molecule with a long serum half-life.[45] The half-life of IgG3-IL2 was examined *in vivo* to determine if fusing IL-2 to IgG3 would result in a molecule with improved pharmacokinetics.

^{125}I-labeled IgG3 or IgG3-IL2 were injected into the peritoneal cavity of mice and the residual radioactivity measured over time (Fig. 7). The fusion protein shows a half-life of 7 hours, reduced compared to IgG3 but greatly increased over the published reports of IL-2.[24] Changing the variable region of the antibody did not dramatically affect the *in vivo* half-life, although linking IL-2 to different locations in the IgG3-IL2 heavy chain did affect its clearance rate (data not shown). Approximately 10% of the injected

protein cleared with a slower half-life, as has been noted with similar proteins.[17,25] IgG3 cleared with an initial half-life of 22 hours. This may represent in part the alpha phase of clearance, as IgG3 has an established half-life of 3-5 days when measured in this way.[26,45]

Biodistribution and toxicity of IgG3-IL2

The *in vivo* fate of an injected protein determines in part its utility as a therapeutic agent. To determine the fate of ^{125}I-labeled IgG3-IL2 *in vivo*, the ^{125}I content of various organs were measured four hours after injection (Fig. 8). The relative concentration within each organ was calculated as described above.

Both IgG3 and IgG3-IL2 permeated organs within and beyond the peritoneal cavity, the site of injection. IgG3 was present at the highest concentration in blood. IgG3-IL2, however, was present at the highest concentration in the thymus and, to a lesser degree, in other organs containing IL-2R bearing cells such as the spleen and lymph nodes. Although present at five times the average concentration of the rest of the animal, the small size of the thymus of adult mice attracted less than 5% of the total injected dose. The majority of the fusion protein was distributed throughout the animal, indicating that it can permeate most tissues.

Mice tolerated large doses of IgG3-IL2 without serious toxicity. Intraperitoneal injection of 100 μg did not result in any noticeable morbidity, but did cause some weight gain. Approximately 48 hours after injection, mice had gained an average (n=3) of 1 gram, or 6% of their total body weight. This increase was transient, and weights returned to normal after 96 hours.

Recovery of IgG3-IL2 from blood

When following the fate of iodinated proteins, there is a concern that the residual label remains associated with the

injected protein. To address this concern, four hours after intraperitoneal injection of ^{125}I-IgG3 or IgG3-IL2, mice were sacrificed, the serum collected and the labeled protein recovered as described above. Greater than 50% of the radioactivity was precipitated from the serum of each mouse using antigen coated Sepharose beads. SDS-PAGE analysis of the precipitated proteins shows that both IgG3 and IgG3-IL2 remain intact as fully assembled H2L2 molecules (Fig. 9A). Reducing the disulfide bonds yields a heavy chain-IL-2 fusion polypeptide unchanged in size, demonstrating that the IL-2 portion of this molecule remains intact after 4 hours in vivo (Fig. 9B). The light chain of the antibody is not visible because labeling conditions approach a ratio of one ^{125}I molecule per protein and do not efficiently label the light chain.

Increased antibody response to IgG3-IL2 bound antigen

To determine if IgG3-IL2 could affect the antibody response to bound antigen, Sepharose beads coated with DNS-BSA were mixed with PBS, anti-dansyl IgG3 or IgG3-IL2 for 1 hour at 4°C. The beads were then injected into the abdomen of mice, and blood was collected periodically to determine the antibody response to DNS-BSA. A second, identical booster injection was delivered on day 70 to all mice. DNS-BSA coated plates were used with isotype specific detecting antibodies in ELISAs to quantitate the response (Fig. 10).

All isotypes and the overall response in the Kappa ELISA show a large increase over the control groups by 20 days after inoculation. In all cases, the concentrations of antigen specific antibodies decreased following the booster injection on day 70, indicating that the antigen coated beads absorbed much of the circulating antibodies.

IgM increases over time and responds to the boost with a further increase. IgA shows a similar pattern, although levels appear to stabilize at 60 days and respond more

dramatically to the boost. IgG1 increases rapidly to a maximal level 25 days after the initial inoculation and begins to decline after 40 days, but responds to the boost with an increase. A similar early maximal response is seen with IgG2, which shows a very large increase compared to the almost undetectable response of the controls at 20 days, a decline after 40 days and no response to the boost. IgG3 increases to 50 days and declines thereafter with only a small response to the boost. The two control groups showed similar antibody response to antigen with only small differences noted in most isotypes. The IgG3 coated beads did, however, stimulate higher levels of IgG1 overall and generate an increased IgM response to boost.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

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CLAIMS:

1. An antibody-cytokine fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody moiety, L is a covalent bond or linking group, and Ck is a cytokine moiety, lymphokine moiety or biologically active fragment thereof.

2. The fusion protein of Claim 1, wherein said antibody moiety is a monoclonal antibody.

3. The fusion protein of Claim 2, wherein said antibody moiety is an anti-dansyl monoclonal antibody.

4. The fusion protein of Claim 1, wherein L is a linking group of from 1 to 10 naturally-occurring amino acids.

5. The fusion protein of Claim 4, wherein L is a linking group of from 1 to 5 naturally-occurring amino acids.

6. The fusion protein of Claim 5, wherein L is a cysteine residue.

7. The fusion protein of Claim 1, wherein Ck is selected from the group consisting of an interleukin, macrophage arming factor, lymphocyte inhibition factor, monocyte chemotactic and activating factor and granulocyte-macrophage colony stimulating factor.

8. A composition comprising:

(a) a fusion protein of the formula (Ab)-L-(Ck), where Ab is an anti-dansyl antibody, L is a covalent bond or a

linking group of from 1 to 10 naturally-occurring amino acids, and Ck represents the amino acid sequence of a cytokine, a lymphokine or a biologically active fragment thereof; and

(b) a dansylated antigen.

9. A method of stimulating an immune response to an antigen, comprising administering an antibody-cytokine fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody moiety, L is a covalent bond or linking group, and Ck is a cytokine moiety, lymphokine moiety or biologically active fragment thereof, to a patient in need thereof in an amount effective to stimulate the immune response of said patient.

10. A method of stimulating an immune response to an antigen, comprising administering an antibody-cytokine fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody moiety, L is a covalent bond or linking group, and Ck is a cytokine moiety, lymphokine moiety or biologically active fragment thereof, to an animal which exhibits an immune response and which has cells having a surface receptor for the Ck moiety of the fusion protein, in an amount effective to stimulate the immune response of said animal.

11. A method for treating a viral, bacterial, fungal or retroviral infection, comprising the method of Claim 9, wherein Ab is an anti-dansyl monoclonal antibody, and the antigen is dansylated and is selected from the group

consisting of a retroviral coat protein, a viral coat protein, an enterotoxin or other bacterial toxin, a fungal protein, an antigenic region of such a protein or toxin and an amino sugar or polysaccharide of a bacterial or fungal cell wall.

12. A method of producing a fusion protein, comprising:

culturing a cell line transformed with a gene encoding a fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody, L is a covalent bond or naturally-occurring amino acid-containing linking group, and Ck is a known cytokine, lymphokine or biologically active fragment thereof, in a medium supporting the growth of the cell line for a length of time sufficient to express the fusion protein, and

recovering the fusion protein from the cell culture.

13. A kit for stimulating an immune response to an antigen, comprising:

(a) a fusion protein of the formula (Ab)-L-(Ck) in a physiologically acceptable carrier, where Ab is an anti-dansyl antibody, L is a covalent bond or a linking group of from 1 to 10 naturally-occurring amino acids, and Ck represents the amino acid sequence of a cytokine, a lymphokine or a biologically active fragment thereof; and

(b) a dansylated antigen in a physiologically acceptable carrier.

14. A method of imaging an IL-2 receptor-bearing tumor, comprising administering a labeled antibody-cytokine fusion protein of the formula:

(Ab)-L-(Ck)

wherein Ab is an antibody moiety, L is a covalent bond or linking group, and Ck is IL-2 or an IL-2R-binding fragment thereof, to a patient in need thereof in an amount effective to detect said tumor.

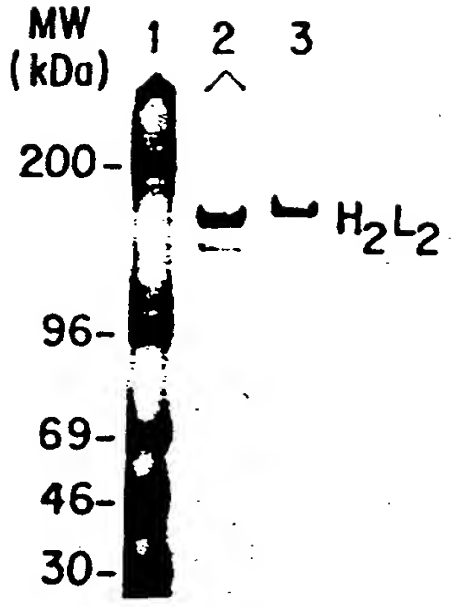

FIG. 1A

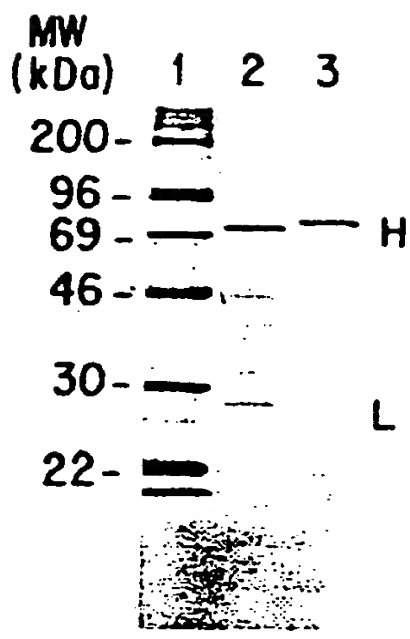

FIG. 1B

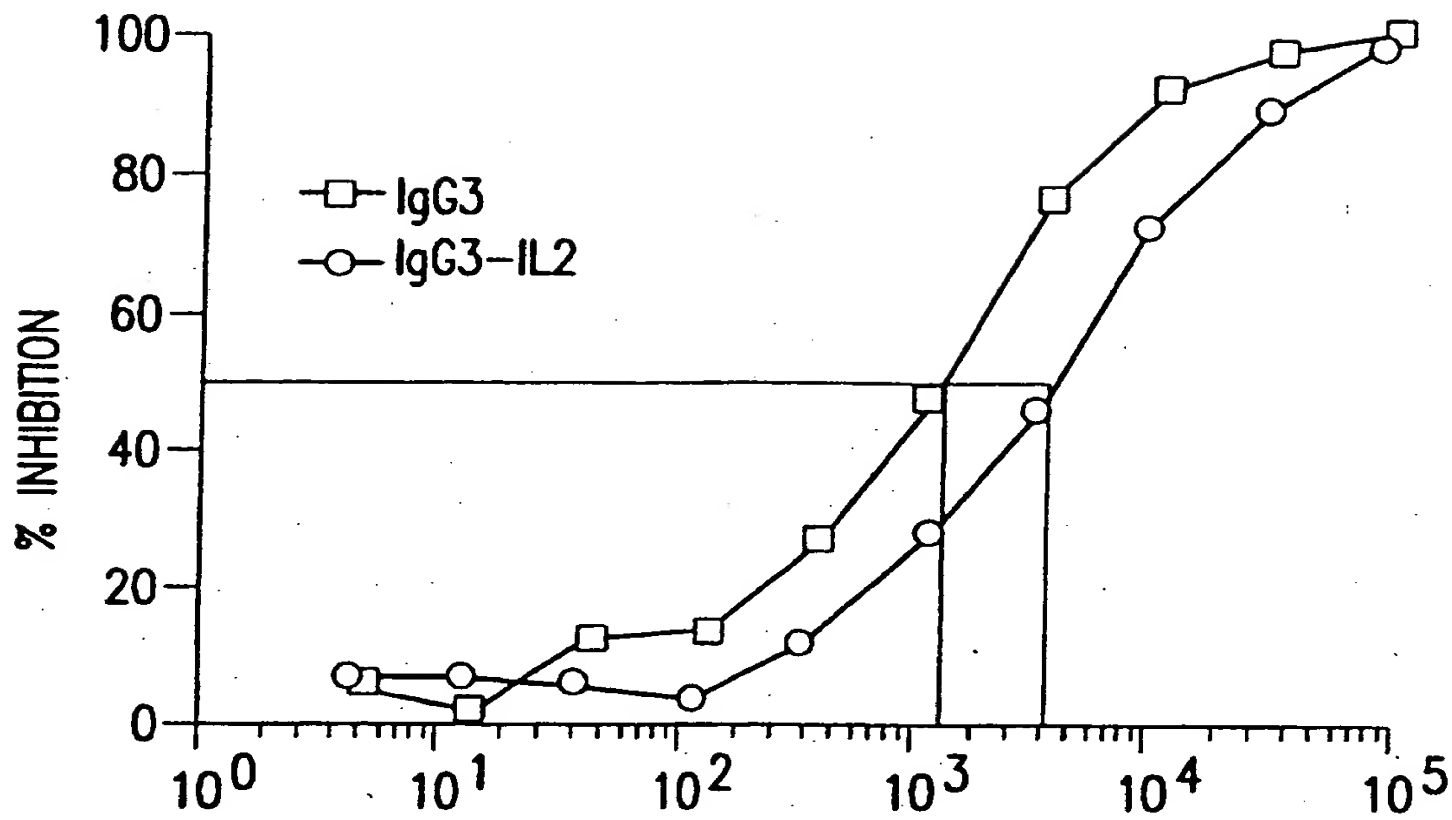
100
80
60
40
20
0
% INHIBITION
IgG3
IgG3-IL2
10^0
10^1
10^2
10^3
10^4
10^5
COLD COMPETITOR (PM)

FIG.2

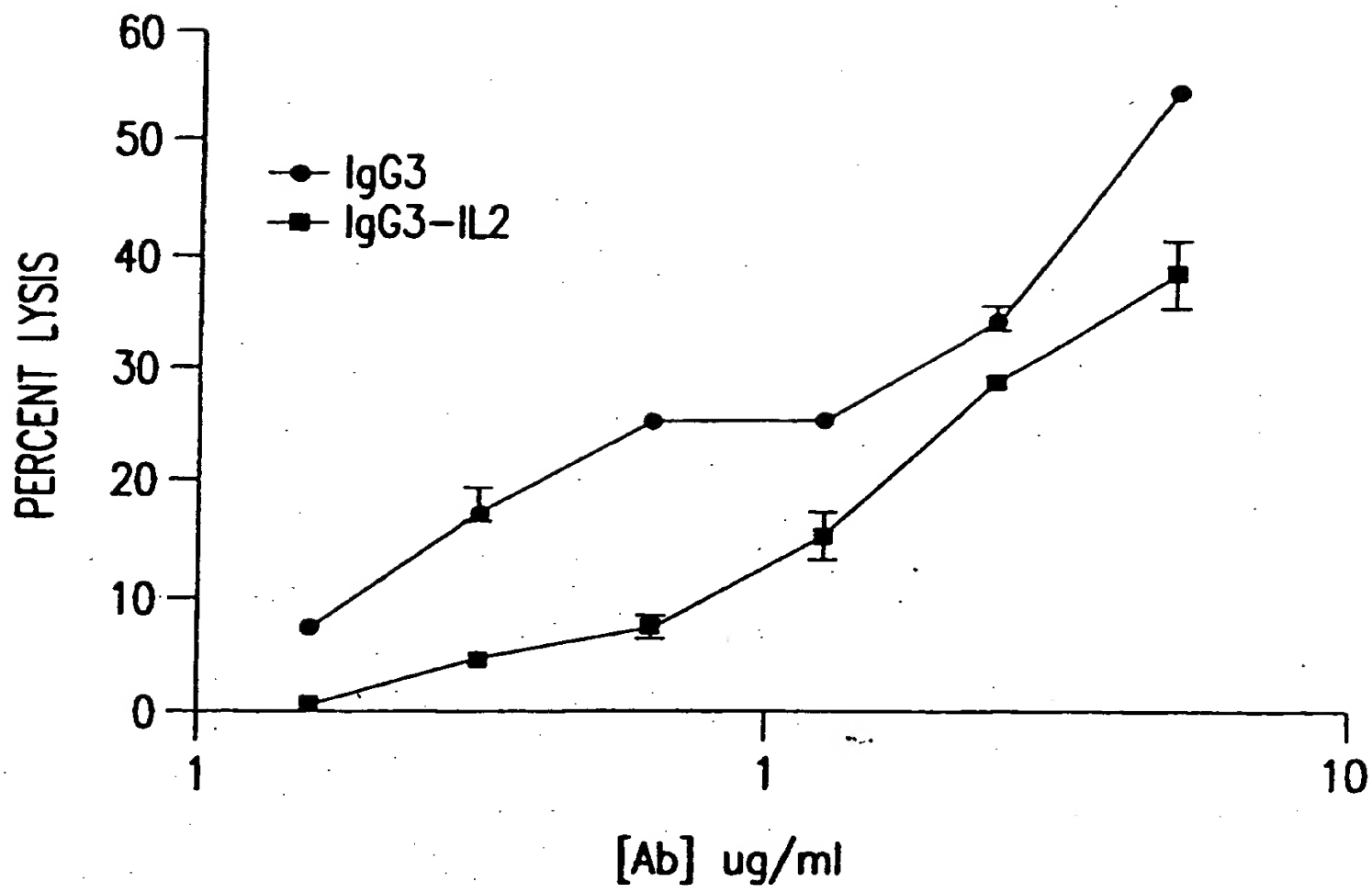

FIG.3

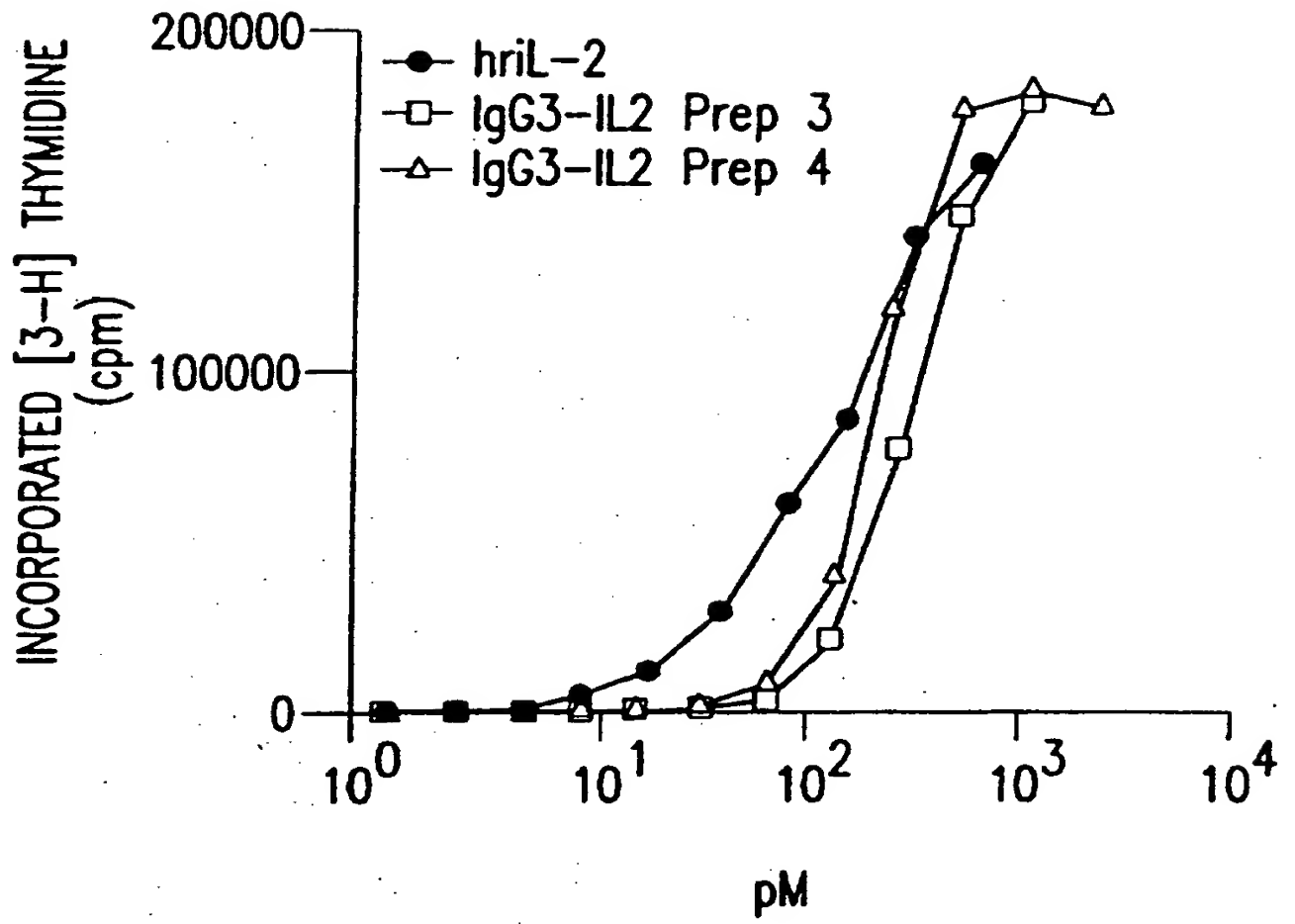
200000
100000
0
hriL-2
IgG3-IL2 Prep 3
IgG3-IL2 Prep 4
INCORPORATED [3-H] THYMIDINE (cpm)
10^0
10^1
10^2
10^3
10^4
pM

FIG.4

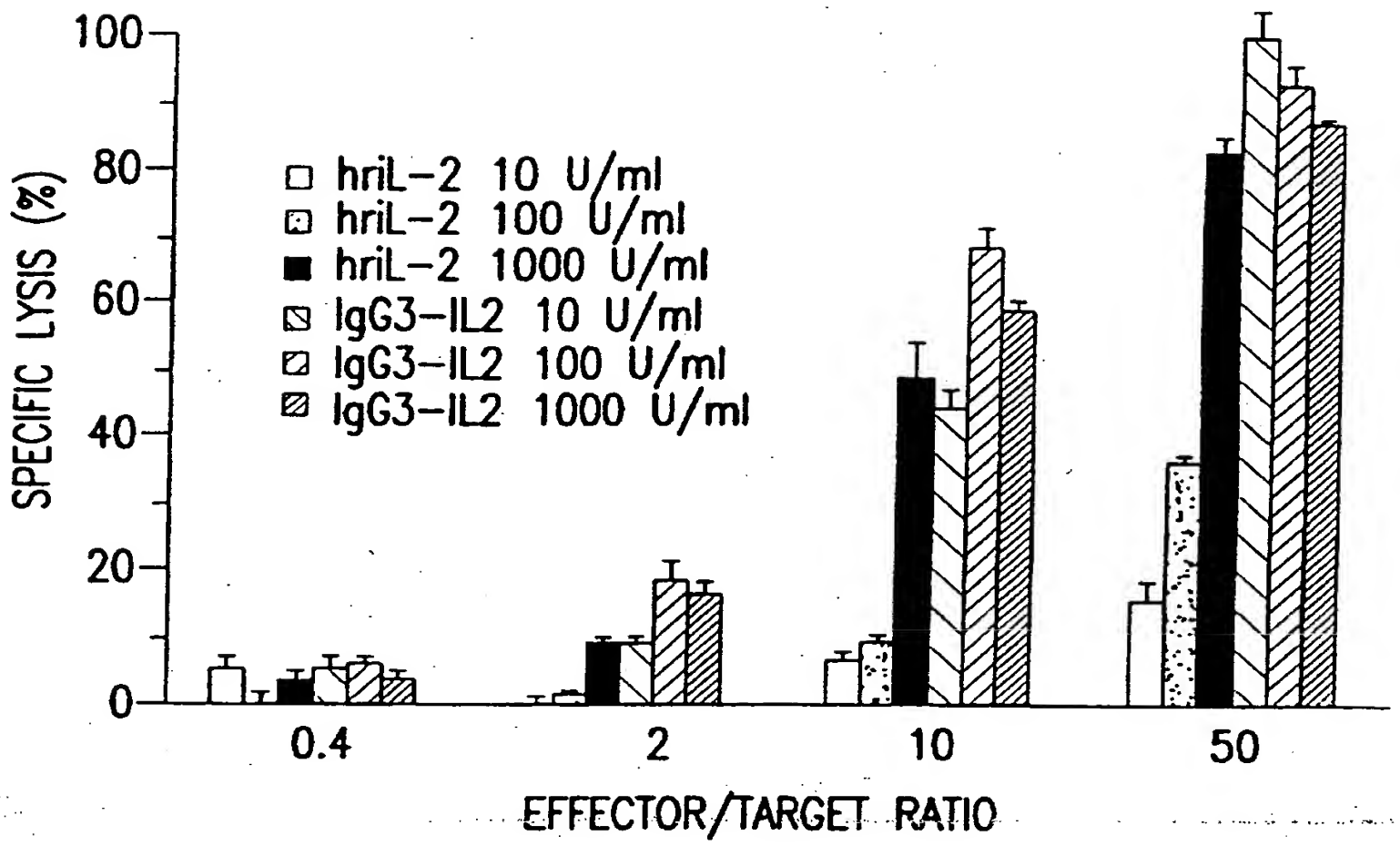
100
80
60
40
20
0
SPECIFIC LYSIS (%)
hriL-2 10 U/ml
hriL-2 100 U/ml
hriL-2 1000 U/ml
IgG3-IL2 10 U/ml
IgG3-IL2 100 U/ml
IgG3-IL2 1000 U/ml
0.4
2
10
50
EFFECTOR/TARGET RATIO

FIG.5

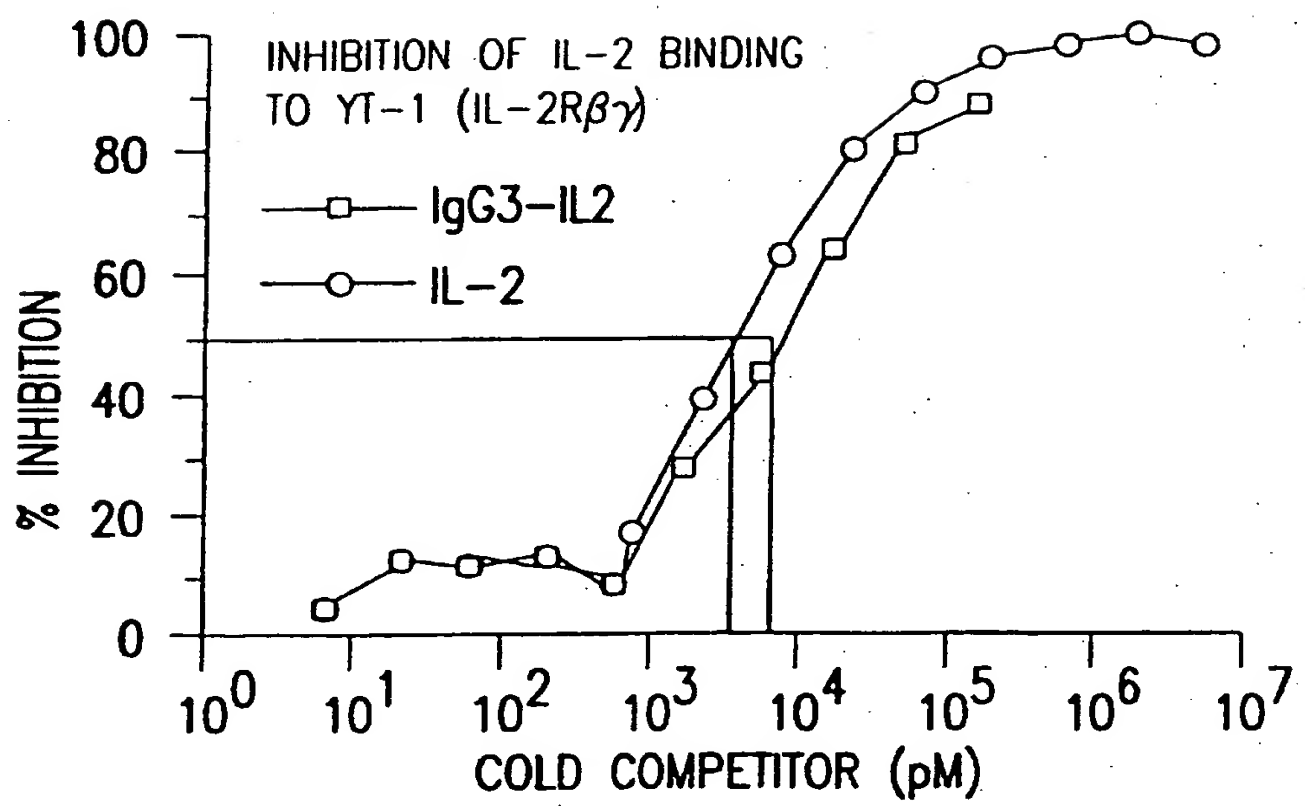
INHIBITION OF IL-2 BINDING
TO YT-1 (IL-2Rβγ)
IgG3-IL2
IL-2
% INHIBITION
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7
COLD COMPETITOR (pM)

FIG.6A

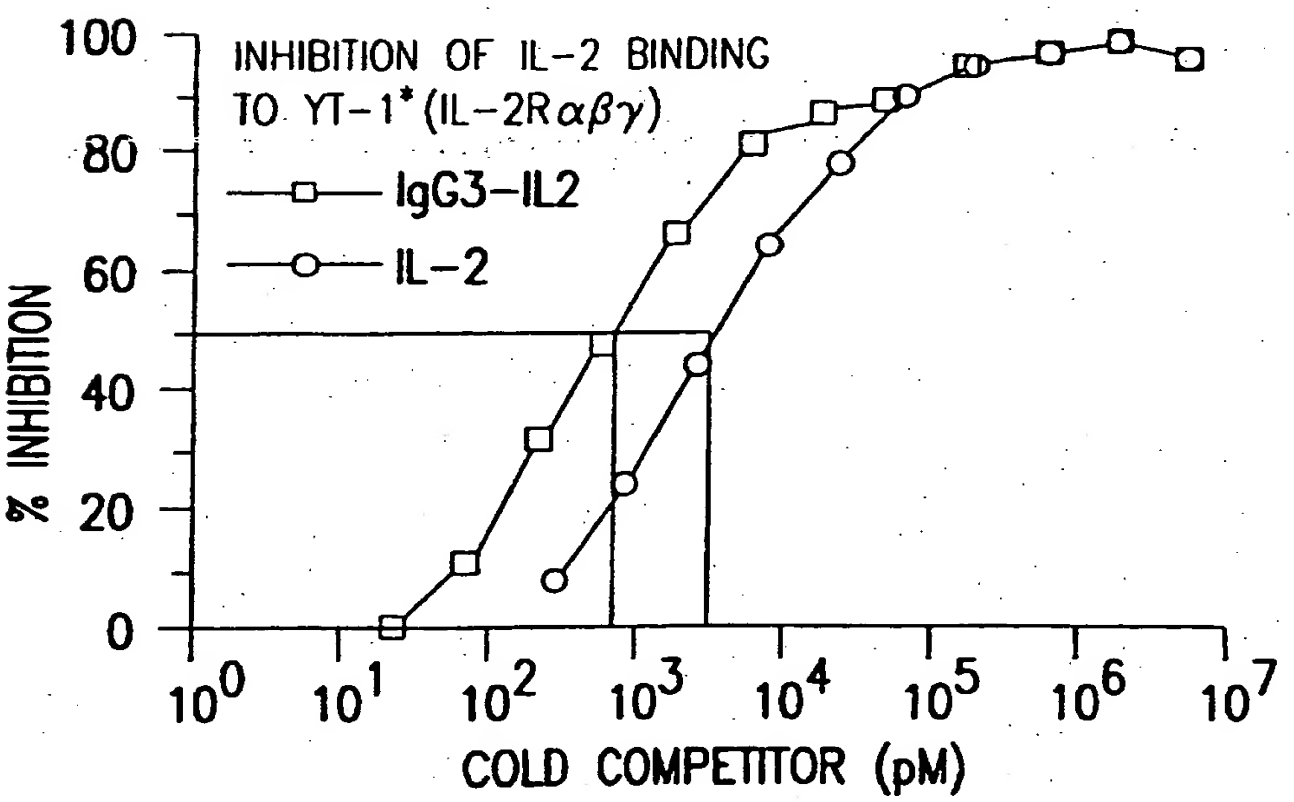
INHIBITION OF IL-2 BINDING
TO YT-1* (IL-2Rαβγ)
IgG3-IL2
IL-2
% INHIBITION
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7
COLD COMPETITOR (pM)

FIG.6B

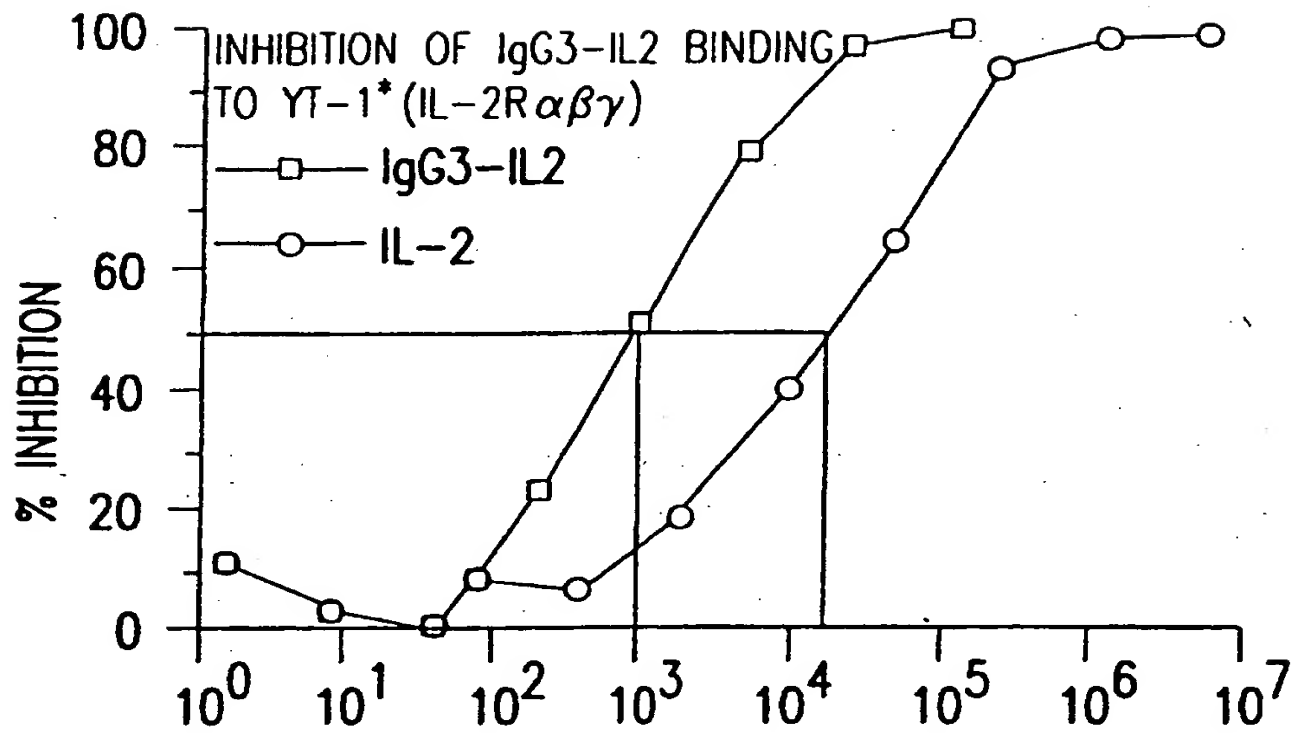
INHIBITION OF IgG3-IL2 BINDING TO YT-1* (IL-2Rαβγ)
IgG3-IL2
IL-2
% INHIBITION
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7
COLD COMPETITOR (pM)

FIG.6C

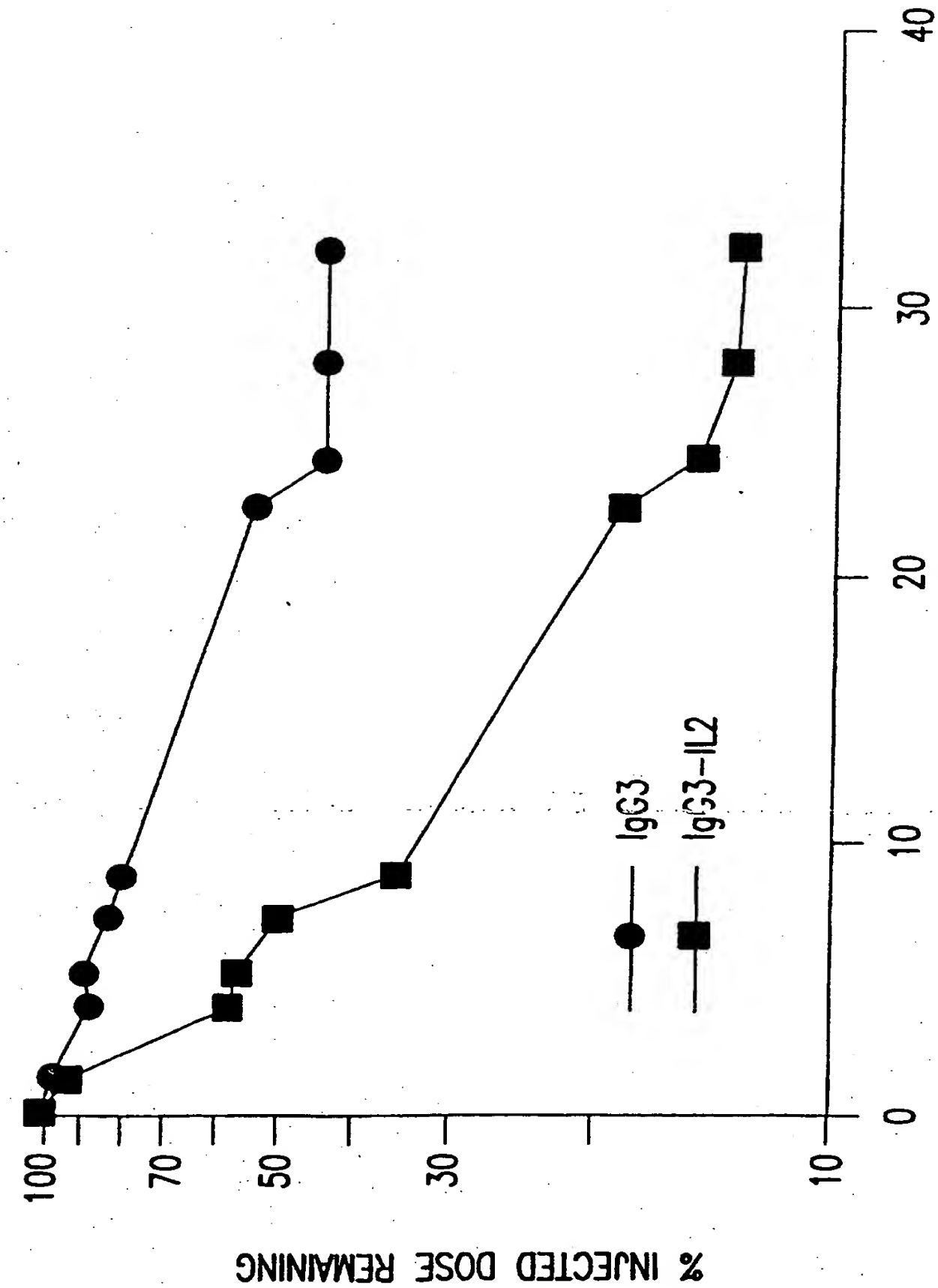
% INJECTED DOSE REMAINING
100
70
50
30
10
0
10
20
40
HOURS
IgG3
IgG3-IL2

FIG.7

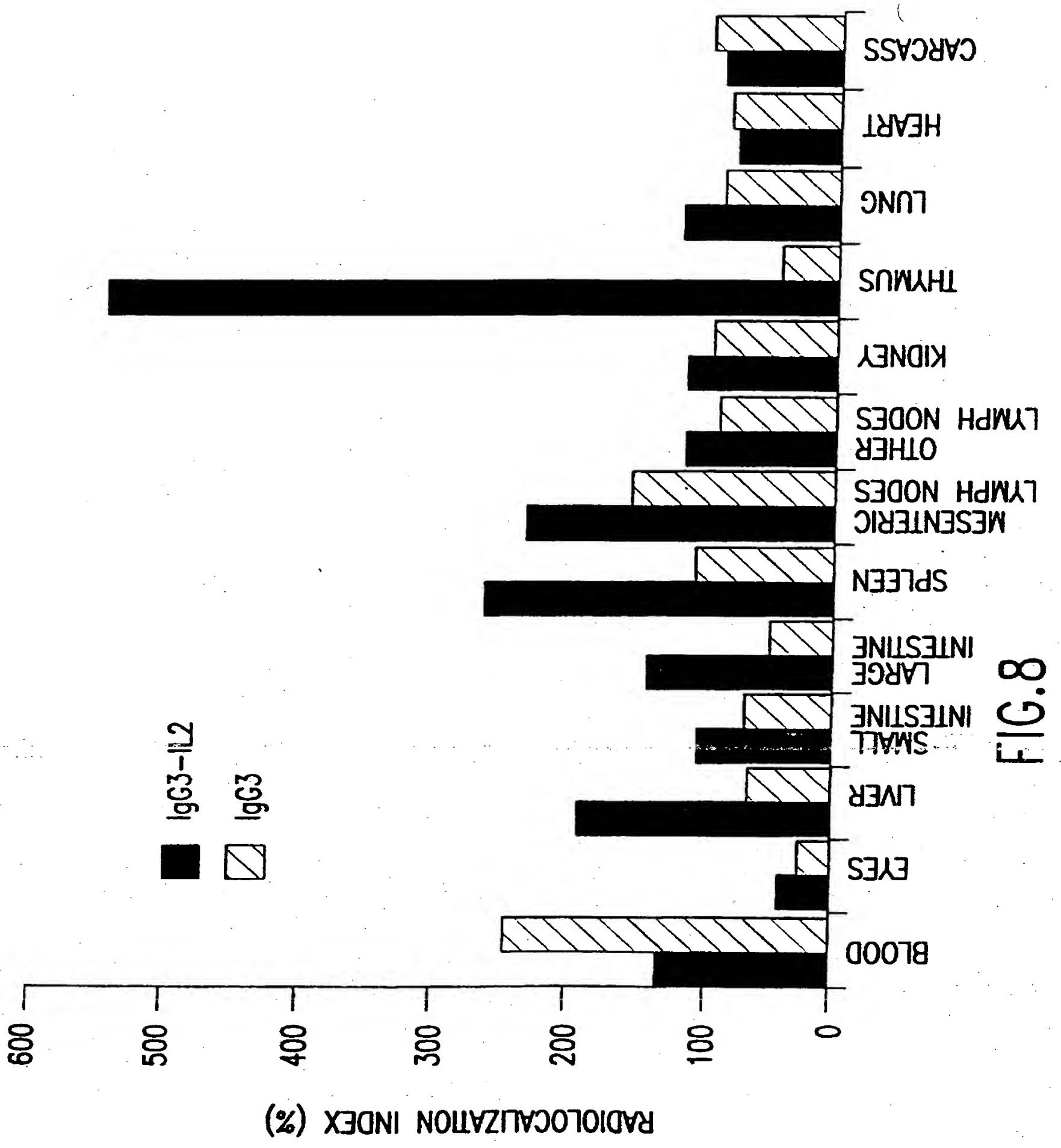
RADIOLOCALIZATION INDEX (%)
600
500
400
300
200
100
0
BLOOD
EYES
LIVER
SMALL INTESTINE
LARGE INTESTINE
SPLEEN
MESENTERIC LYMPH NODES
OTHER LYMPH NODES
LUNG
HEART
CARCASS
IgG3
IgG3-IL2

FIG.8

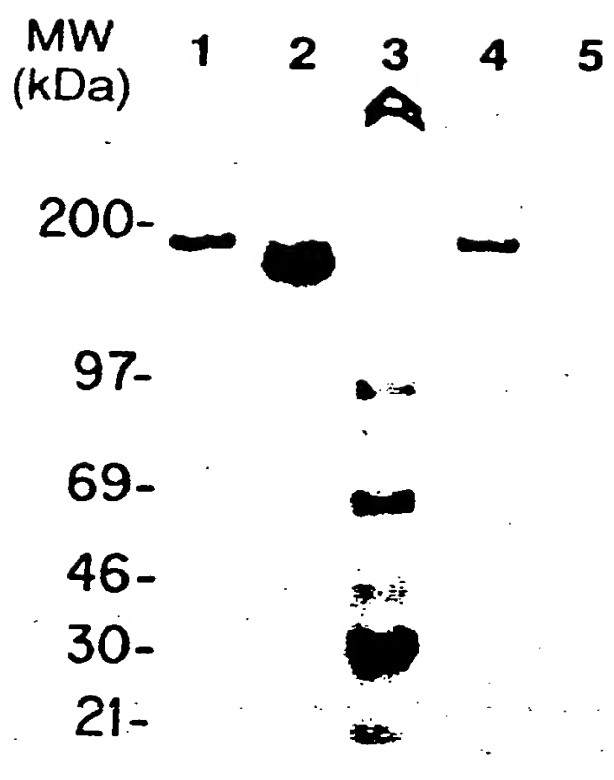

FIG.9A

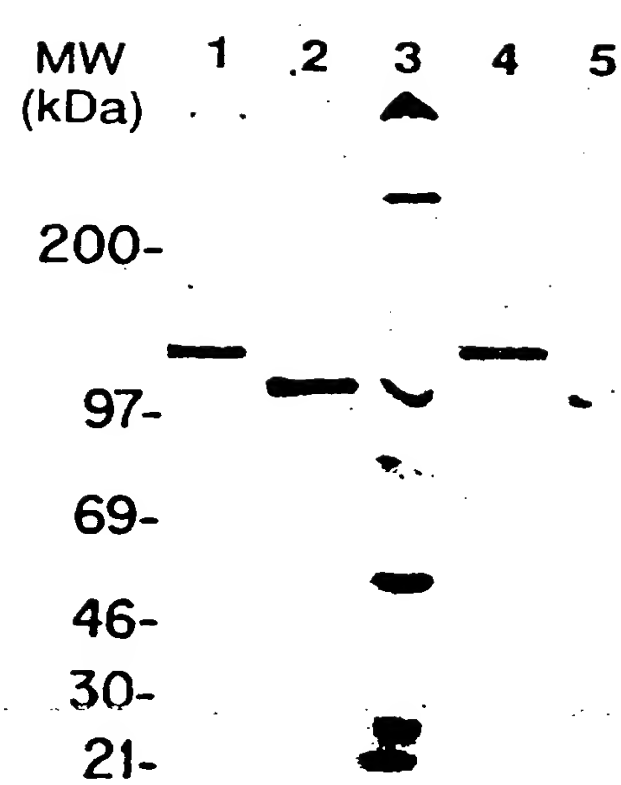

FIG.9B

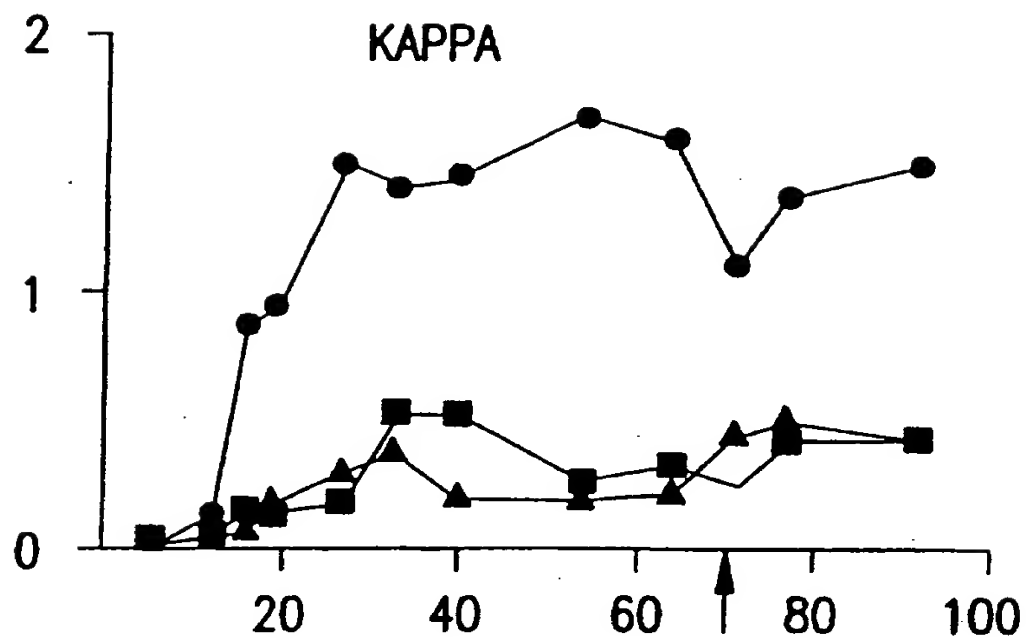

FIG.10A

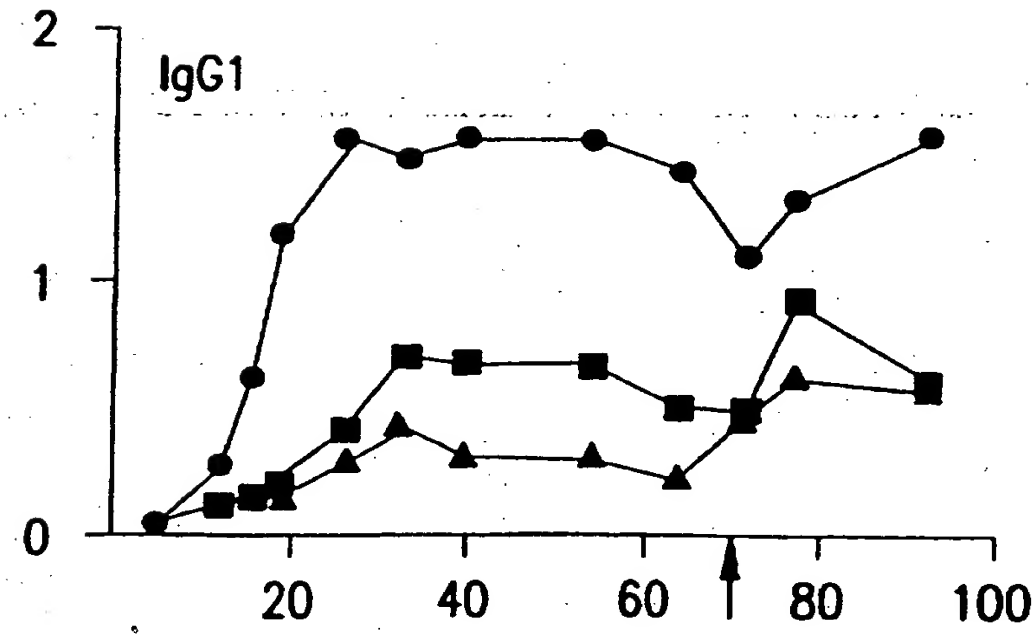

FIG.10B

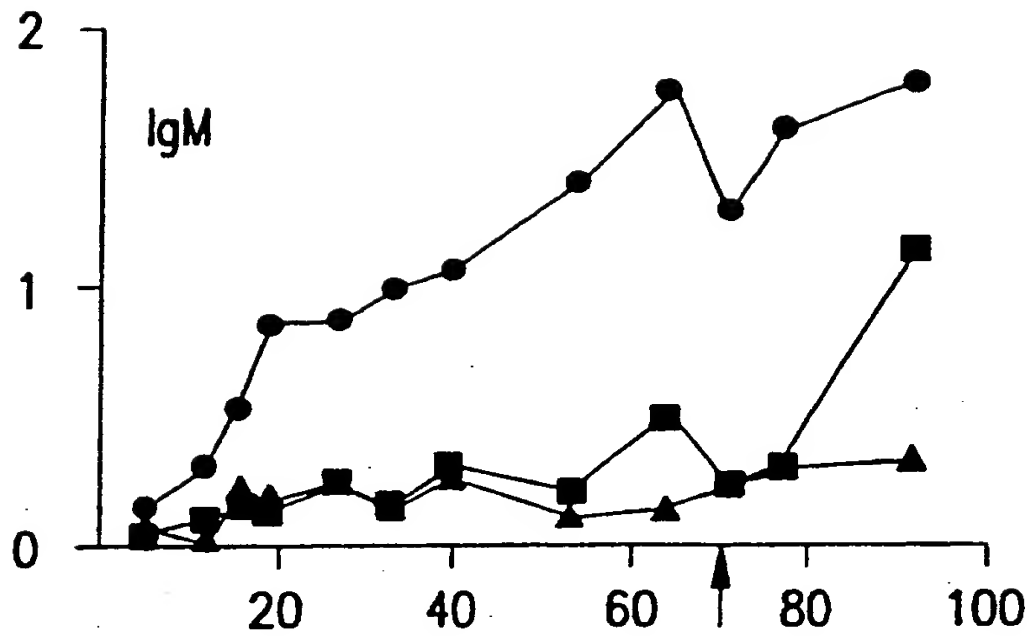

FIG.10C

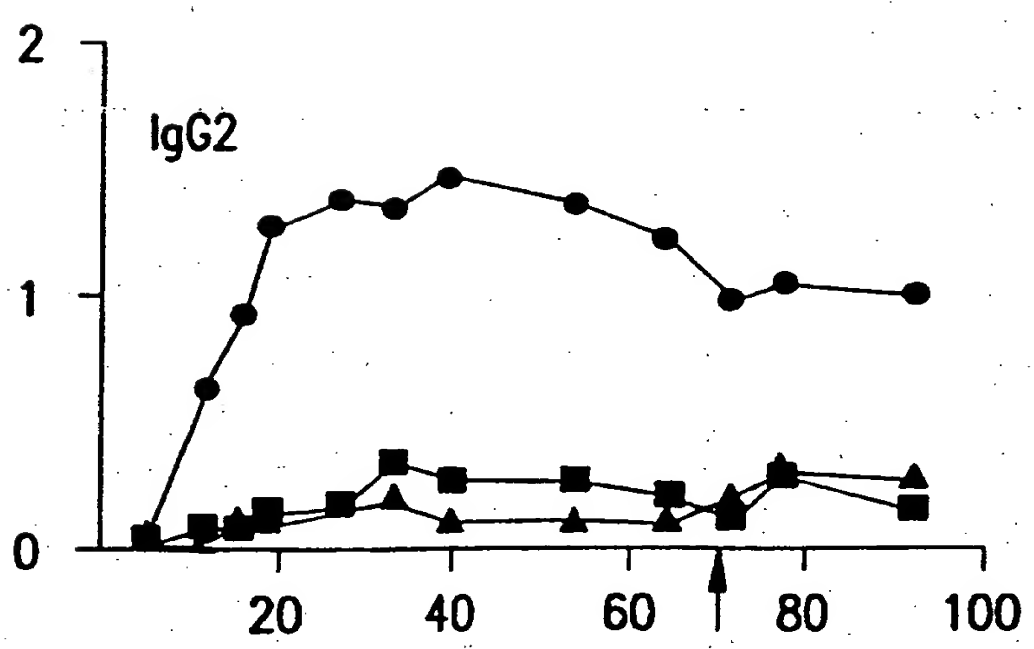

FIG.10D

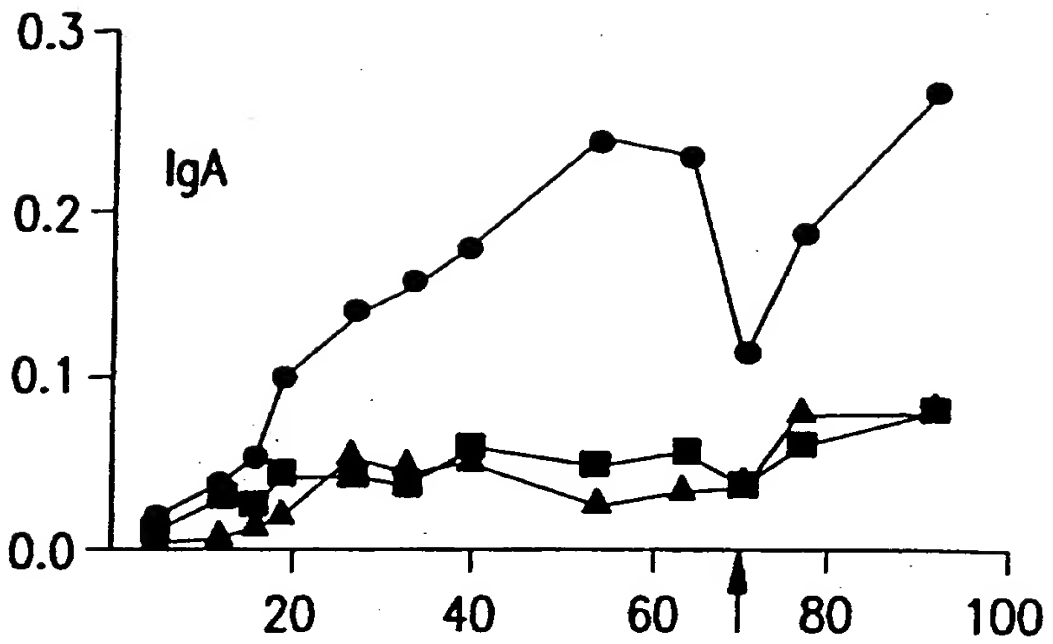

FIG.10E

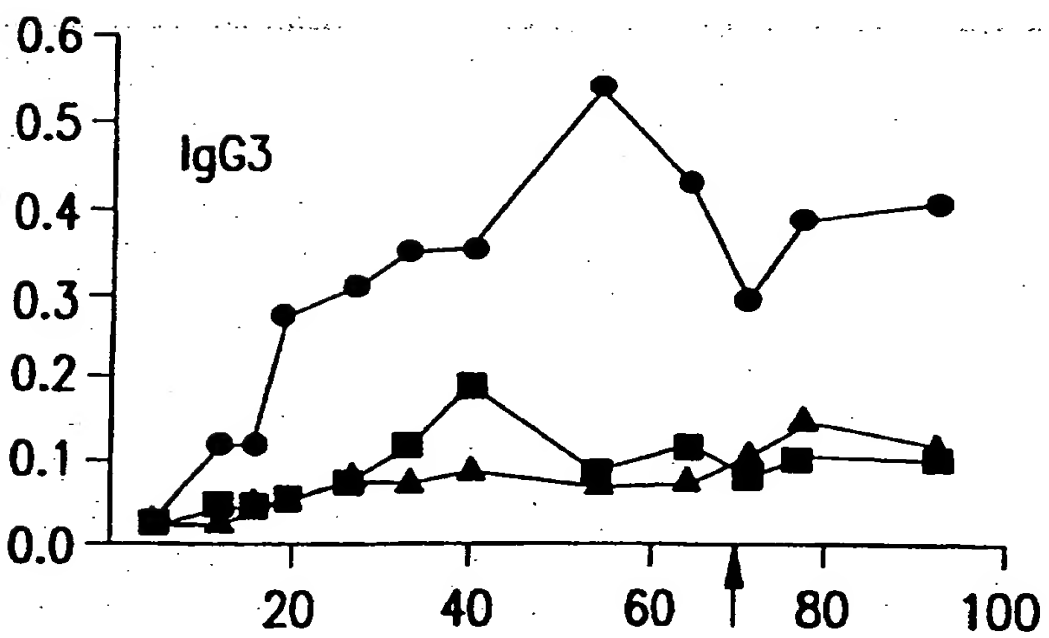

FIG.10F